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(71) Applicant: **CYTEL CORPORATION**
3525 John Hopkins Court
San Diego, CA 92121 (US)

(72) Inventor: **Vitiello, Maria A.**
8522 High Avenue
La Jolla, CA 92037 (US)
Inventor: **Chesnut, Robert W.**
1473 Kings Cross Drive
Cardiff by the Sea, CA 92007 (US)

(74) Representative: **Fisher, Adrian John**
CARPMAELS & RANSFORD 43 Bloomsbury
Square
London WC1A 2RA (GB)

(54) **HLA-restricted hepatitis B virus CTL epitopes.**

(57) Cytotoxic T lymphocyte-stimulating peptides induce HLA-restricted responses to hepatitis B virus antigens. The peptides, derived from CTL epitopic regions of both HBV surface and nucleocapsid antigens, are particularly useful in the treatment and prevention of HBV infection, including the treatment of chronically infected HBV carriers. The peptides can be formulated as HBV vaccines and pharmaceutical compositions, such as lipid-containing compositions for enhancing the HLA-restricted CTL responses. The peptides are also useful in diagnostic methods, such as predicting which HBV-infected individuals are prone to developing chronic infection.

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Related Applications

The present application is a continuation-in-part of U.S. Serial No. 07/874,491, filed April 27, 1992, which is a continuation-in-part of U.S. Serial No. 07/827,682, filed January 29, 1992, which is a continuation-in-part of USSN 07/749,568, filed August 26, 1991, each of which is incorporated herein by reference.

Background of the Invention

The hepatitis B virus (HBV) is not believed to be directly responsible for damage to hepatocytes, despite its predilection for infecting such cells. Rather, non-viral host factors are implicated in the pathogenesis of hepatitis. It is suspected that a variation in immune responsiveness to HBV infection may account for the wide diversity of syndromes associated with HBV infection.

Following an acute HBV infection, approximately 90% of affected adults recover without sequelae and develop immunity to the virus, although the clinical course of the infection during the acute phase can itself be quite variable. In 5-10% of infected adults, a chronic HBV infection becomes established. Chronic HBV infection can range from asymptomatic carrier state to continuous hepatocellular necrosis and inflammation, and in some instances may lead to hepatocellular carcinoma. Children exposed to HBV infection, particularly those less than one year old, often develop chronic infection and represent the major source of chronic infection. Worldwide, nearly 200 million people are chronically infected with HBV. And finally, in a small percentage of HBV infections (0.1 -0.5%) a fulminant hepatitis results in such extreme cell death in the liver that fewer than one-fifth to one-third of these patients survive.

The immune response to hepatitis B virus is as complex as the disease. A variety of humoral and cellular responses have been identified to different regions of the HBV nucleocapsid core and surface antigens. T cell mediated immunity, particularly involving class I major histocompatibility complex (MHC)-restricted cytotoxic T lymphocytes (CTL), is believed to play an important role in resistance to hepatitis as well as several other viral infections. CTL recognize antigen in the form of small peptides in association with the class I histocompatibility molecules. The antigen-specific CTL, when stimulated, can secrete mediators which inhibit viral replication and eliminate infected cells, thereby contributing to an individual's recovery from the infection. Although studies suggest that the T cell repertoire of class I-restricted responses is focused on a limited number of discrete immunodominant epitopes of a viral protein (Braciale et al., *Proc. Natl. Acad. Sci. USA* 86:277-281 (1989)), for many viruses, including the hepatitis viruses and particularly HBV, few epitopes have been identified. See also Barnaba et al., *Nature* 345:258 (1990) have identified an A11 restricted epitope while Jin et al., *J. Exp. Med.* 168:293 (1988) have identified an A3 restricted epitope. Aichele et al., *J. Exp. Med.* 171:1815-1820 (1990), have demonstrated induction *in vivo* of an antiviral CTL response in an MHC class-I dependent fashion with a peptide from the nucleoprotein of lymphocytic choriomeningitis virus. Recently, Kast et al., *Proc. Natl. Acad. Sci. USA* 88:2283-2287 (1991), described the stimulation of Sendai virus-specific CTL *in vivo* using free synthetic peptide derived from the nucleoprotein to confer protection against subsequent viral challenge.

It has been suggested that hepatocyte injury during HBV infection may be mediated by an HLA class I-restricted CTL response to HBV antigen. In attempting to define the CTL response to HBV, it has been shown that peripheral blood lymphocytes from patients with acute and chronic HBV may be able to kill autologous hepatocytes *in vitro*, but the specificity of the cytolytic activity, its HLA restriction elements, and cellular phenotype were not established. See, Mondelli et al., *J. Immunol.* 129:2773 (1982) and Mondelli et al., *Clin. Exp. Immunol.* 6:311 (1987). More recently, Moriyama et al., *Science* 248:361-364 (1990), reported that the HBV major envelope antigen was expressed at the hepatocyte surface in a form recognizable by MHC class I-restricted, CD8⁺ cytotoxic T lymphocytes, and by envelope-specific antibodies. However, the HBV epitopic regions responsible for HBV-specific CTL activity were not identified.

The requirement for lymphokines such as IL-2 in the generation of CD8⁺ CTL is well established, although the need for activation of CD4⁺ T helper cells to provide these lymphokines remains somewhat controversial. While the concept of linked T helper-B cell recognition for antibody production has been firmly defined, there is no compelling evidence for linked T helper-CTL recognition for the *in vivo* induction of CD8⁺ CTL. See, e.g., Buller et al., *Nature* 328:77-79 (1987); Sarobe et al., *Eur. J. Immunol.* 21:1555-1558 (1991); and Cassell and Forman, *Annals N.Y. Acad. Sci.* :51-60 (1991).

Individuals chronically infected with HBV are at risk of developing liver cirrhosis and/or hepatocellular carcinoma, and constitute an extremely large reservoir for spreading the disease. It would be desirable to stimulate the immune systems of those chronically infected to respond to appropriate HBV antigens and eliminate their infections, or to be able to prevent the evolution from an acute HBV infection to the chronic stage. Further, as the presently approved HBV vaccines provide only about 90% protection among those immunized, it is desirable to improve the existing vaccines by increasing or diversifying the immunogenicity of the vaccines to elicit

a more effective immunity. Means are also needed for predicting which patients with acute HBV infection are likely to develop chronic HBV infection, so that appropriate treatment and precautions can be implemented earlier. Quite surprisingly, the present invention fulfills these and other related needs.

5 Summary of the Invention

The present invention provides peptides which induce MHC class I-restricted CTL responses against HBV antigen. The peptides of interest are derived from sequences of the HBV surface antigen or nucleocapsid proteins. In certain embodiments the peptides comprise from six to about thirty amino acids and contain at least one epitope which is capable of inducing a MHC class I-restricted cytotoxic CTL response to HBV where the epitope(s) is within the HBV envelope antigen sequence designated 799.10 (HBenv₃₄₉₋₃₆₈) [Seq. ID No. 2] Leu-Ser-Pro-Thr-Val-Trp-Leu-Ser-Val-Ile-Trp-Met-Met-Trp-Tyr-Trp-Gly-Pro-Ser-Leu or 799.09 (HBenv₃₂₉₋₃₄₈) [Seq. ID No. 7] Ala-Ser-Ala-Arg-Phe-Ser-Trp-Leu-Ser-Leu-Leu-Val-Pro-Phe-Val-Gln-Trp-Phe-Val-Gly (for subtypes ayw and adw). Other peptide embodiments comprise from six to thirty amino acids and have at least seven amino acids from the HBenv sequence 799.08 (HBenv₃₀₉₋₃₂₈) [Seq. ID No. 1] Asn-Cys-Thr-Cys-Ile-Pro-Ile-Pro-Ser-Ser-Trp-Ala-Phe-Gly-Lys-Phe-Leu-Trp-Glu-Trp (for subtype ayw). In yet other embodiments a peptide is derived from HBV core antigen sequence region designated 802.03 (HBc₉₁₋₁₁₀) [Seq. ID No. 3], having the sequence Thr-Asn-Met-Gly-Leu-Lys-Phe-Arg-Gln-Leu-Leu-Trp-Phe-His-Ile-Ser-Cys-Leu-Thr-Phe (subtype ayw). The peptides of the invention can be optionally flanked and/or modified at one or both of the N- and C-termini, as desired, and substitutions, deletions and additions may be made to the peptide as long as the ability of the peptide to stimulate HBV CTL activity or act as an analog antagonist is not substantially adversely affected.

In the various peptide embodiments it will be understood that the peptides can be covalently linked, e.g., via polymerization or conjugation, each to itself to form larger homopolymers, or with different peptides to form heteropolymers. In some instances peptides will be combined in a composition as an admixture and will not be linked. The CTL inducing peptide can also be linked to a lipid-containing molecule capable of enhancing a T lymphocyte response, or may be linked to a T helper peptide which induces a T-helper cell response, or may be linked to both a lipid-containing molecule and a T helper peptide, for example. Linkage to a lipid or a T-helper peptide may be either at the amino or carboxy termini.

Compositions are provided which comprise a peptide of the invention formulated with an additional peptide, a liposome, an adjuvant and/or a pharmaceutically acceptable carrier. Thus, pharmaceutical compositions can be used in methods of treating acute HBV infection, particularly in an effort to prevent the infection from progressing to a chronic or carrier state. Methods for treating chronic HBV infection and HBV carrier states are also provided, where the pharmaceutical compositions are administered to infected individuals in amounts sufficient to stimulate immunogenically effective CTL responses against HBs and HBc epitopes. For treating these infections it may be particularly desirable to combine the peptides which induce MHC class I restricted CTL responses against HBV antigen with other peptides or proteins that induce immune response to other HBV antigens. To treat individuals with chronic or carrier state infections the compositions may be administered with an initial dosage followed by a boosting dosage over a period of time, as necessary to resolve or substantially mitigate the infection.

Vaccine compositions for preventing HBV infection, including preventing development of chronic HBV infection from an acute infection, are also provided. The vaccine compositions comprise an immunogenically effective amount of a HBV peptide which induces a MHC class I restricted CTL response. In the case of HLA-A2 haplotype individuals, the peptide can be derived from any of peptides 799.08 (HBenv₃₀₉₋₃₂₈) [Seq. ID No. 1], 799.09 (HBenv₃₂₉₋₃₄₈) [Seq. ID No. 7], 799.10 (HBenv₃₄₉₋₃₆₈) [Seq. ID No. 2], and/or 802.03 (HBc₉₁₋₁₁₀) [Seq. ID No. 4], and will typically further comprise an adjuvant, e.g., incomplete Freund's adjuvant, aluminum hydroxide, or the like. To achieve enhanced protection against HBV, the vaccine can further comprise additional components to elicit protective cellular and/or antibody responses to HBV antigens. In preferred embodiments the CTL inducing peptides are administered with one or more T helper peptides which contain T helper epitopes. Selection of the T helper peptide depends upon whether the vaccine composition containing a T helper peptide is administered prophylactically or therapeutically. In the case of prophylactic administration the T helper peptide will be one or more HBV peptides derived from the HBV envelope or core or proteins derived from other organisms such as tetanus toxoid, influenza, parainfluenza, malaria, Epstein Barr virus, herpes simplex and others known to one of ordinary skill in the art. In the case of therapeutic administration the T helper epitope will preferably be peptides selected from proteins derived from infectious agents other than HBV. Several examples of T helper peptide are HBc128-140, HBc 1-20, HBc 50-69 and HBc 111-125 tetanus toxoid 830-843 and influenza 307-319. The T helper peptides may be administered simultaneously or separately with the

CTL-inducing peptide, but preferably the CTL and T helper peptides are linked. The linkage of the peptides may comprise a spacer molecule, such as amino acids residues, e.g., alanine or other relatively neutral residues.

In yet other embodiments the invention relates to methods for diagnosis, where the peptides of the invention are used to determine the presence in an individual of lymphocytes which are capable of a cytotoxic T cell response to HBV surface or nucleocapsid antigen. The absence of such cells determines whether the individual of interest is susceptible to developing chronic HBV infection. Typically the lymphocytes are peripheral blood lymphocytes and the individual of interest is suffering from an acute HBV infection.

Brief Description of the Drawings

Fig. 1 depicts the results of induction of HBV peptide-specific A2.1-restricted CTL by priming A2.1/K^b transgenic mice with syngeneic spleen cells "loaded" with HBV. Panels A-D: Splenocytes from HBV-primed transgenic mice were restimulated *in vitro* with four mixtures of syngeneic LPS blasts each coated with one of 13 different peptides. After 9 days effector cells were assayed for lytic activity against ⁵¹Cr labelled Jurkat A2.1/K^b target cells in the presence or absence of the four different peptide mixtures used for induction. Panels E-M: Effector cells raised against the four different peptide mixtures were restimulated *in vitro* against the same peptide mixtures and assayed for lytic activity against ⁵¹Cr labelled Jurkat A2.1/K^b target cells in the presence or absence of the individual peptides.

Fig. 2 illustrates the HBV peptide specificity of A2.1 transgenic CTL. Transgenic CTL raised from HBV-primed transgenic mice and restimulated *in vitro* twice with one of the four different peptide mixtures were restimulated with individual HBV peptides and assayed for lytic activity on ⁵¹CR labelled Jurkat target cells in the presence or absence of the HBV peptides used for the restimulation.

Fig. 3 illustrates the results of induction of HBV peptide-specific A2.1-restricted CTL by priming A2.1/K^b transgenic mice with HBV in IFA. A. Splenocytes from HBV-primed transgenic mice were restimulated *in vitro* with syngeneic LPS blasts coated with HBV peptides. After 6d, effector cells were assayed for lytic activity against ⁵¹Cr labelled Jurkat A2.1/K^b target cells in the presence or absence of the appropriate HBV peptide. Each panel represents the CTL activity induced by the indicated target peptide.

Fig 4. The effector CTL of Fig. 3 were restimulated with peptide coated LPS blasts followed at a one week interval by restimulation with peptide coated Jurkat A2.1/K^b cells. Six days after the last restimulation, effector cells were assayed for cytolytic activity against ⁵¹Cr labelled Jurkat A2.1/K^b target cells in the absence or presence of the peptide used for the restimulation, plus related peptides. Each panel represents the CTL activity induced by the peptide indicated in the corresponding panel of Fig. 3. The target peptides are indicated in each panel.

Fig. 5 illustrates that no HBc18-27-specific CTL response is detected in mice primed with the HBc 875.23 T helper epitope alone. Animals were primed subcutaneously with 100 µg of 875.23 (T helper epitope) in Complete Freund's Adjuvant (CFA) followed 9 days later (subcutaneously) with IFA alone. Splenocytes were removed 3 weeks later, cultured for 6 days in the presence of LPS-blasts that had been incubated with the CTL epitope (875.15), 100 µg for 2 hrs before being washed and added to the culture as a source of antigen presenting cells. The presence of HBc 18-27 (875.15)-specific CTL was determined using a standard 6 hr ¹⁵Cr release assay with Jurkat A2.1/K^b cells as targets.

Fig. 6 illustrates that no HBc 18-27-specific CTL response was detected when mice were primed with HBc18-27 (875.15) in IFA. Experimental protocol was similar to that described in Fig. 5, except that mice received 100 µg of peptide 875.15 subcutaneously in IFA rather than IFA alone for *in vivo* CTL priming.

Fig. 7 illustrates that HBc18-27-specific CTL response was detected in 50% of the mice primed with HBc T helper peptide (875.23) mixed with HBc CTL inducing peptide (875.15) at a 1 to 1 ratio. The experimental protocol was similar to that described in Figs. 5 and 6.

Fig. 8 illustrates that HBc-specific (875.15) CTL activity was detected in mice primed with peptide 902.01 in which the HBc T helper and CTL inducing peptide were linked via a peptide bond. Experimental protocol was similar to that in Figs. 5 and 6.

Fig. 9 illustrates that the greatest HBc18-27 (875.15)-specific CTL activity was detected in mice primed with peptide 902.02 in which the HBc T helper and CTL epitopes were linked via peptide bonds using an exemplary spacer such as alanine-alanine-alanine. Protocol was similar to that in Figs. 5 and 6.

Fig. 10 illustrates that previous priming of helper T cells was not required for *in vivo* priming of HBc 18-27-specific CTL responses using peptide 902.01 and 902.02. CTL response is shown from animals primed subcutaneously with peptide 902.01 (Fig. 10A) or 902.02 (Fig. 10B) alone without the previous priming with peptide 875.23 in CFA.

Fig. 11 illustrates the induction of HBenv₃₆₀₋₃₆₈ specific CTL response. A2.K^b transgenic mice were injected with 100 microliters of an emulsion (IFA) of 100 mg HBenv360-368 and 100 mg HBc128-140. Three weeks later, splenocytes were restimulated with syngeneic LPS blasts coated with peptide HBenv360-368. Effector cells were assayed for cytotoxicity against ⁵¹Cr labeled Jurkat A2/K^b target cells in the presence or absence of HBenv 360-368.

Fig. 12 illustrates the induction of a CTL response specific for HBc 18-27 by priming with a peptide containing HBc 18-27 linked to tetanus toxoid 830-843 (human helper T cell epitope). Effector cells were assayed against ⁵¹Cr labeled Jurkat A2-1/K^b target cells in the presence or absence of HBc 18-27; Jy target cells in the presence or absence of HBc 18-27 and Jy cells that had been transfected with HBV core.

Fig. 13 illustrates the minimal sequence for CTL recognition within HBV env 329-348 peptide (799.09). CTL lines 110 and 113 were derived from splenocytes obtained from A2K^b transgenic mice primed subcutaneously with HBV virus in IFA and in vitro activated with 799.09 coated stimulator cells. 799.09 specific CTL lines 110 and 113 were assayed for lytic activity in a 6 hr ⁵¹Cr release assay using JA2K^b cells as targets in the presence of 799.09 peptide truncations (Panel A = 799.09 N-terminus truncations; Panel B = 799.09 overlapping 9 mers and 10 mers).

Description of the Specific Embodiments

The present invention provides peptides derived from the HBV envelope antigen (HBenv, also referred to as surface antigen, or HBs) and nucleocapsid core (HBc) protein sequences for use in compositions and methods for the treatment, prevention and diagnosis of HBV infection. The peptides are capable of inducing MHC HLA class I-restricted CTL responses to HBV infected cells. The stimulated CTL, which secrete lymphokines (e.g., gamma interferon) and liberate products (e.g., proteolytic enzymes such as serine esterases) that inhibit viral replication in infected autologous cells or transfected cells, with or without cell killing, are able to interrupt or substantially prevent chronic HBV infection. In many instances the combination of an effective cytotoxic T cell response and a protective antibody response to selected HBV antigens will be most effective in preventing or terminating an HBV infection.

In preferred embodiments the peptides of the invention are derived from within the HBV surface antigen or the nucleocapsid polypeptides, core and precore. In more preferred embodiments described herein the CTL-inducing peptides are derived from the region of HBenv₃₀₉₋₃₂₈ (peptide 799.08), HBenv₃₂₉₋₃₄₈ (peptide 799.09) HBenv₃₄₉₋₃₆₈ (peptide 799.10), or the region HBc₉₁₋₁₁₀ (peptide 802.03), where the numbering is according to Galibert et al., *Nature* 281:646 (1979), which is incorporated herein by reference.

By "CTL inducing peptide" or "oligopeptide" of the present invention is meant a chain of at least four amino acid residues, preferably at least six, more preferably eight to ten, sometimes eleven to fourteen residues, and usually fewer than about thirty residues, more usually fewer than about twenty-five, and preferably fewer than fifteen, e.g., eight to fourteen amino acid residues derived from selected epitopic regions of the HBenv or HBc proteins, or such other epitopic regions of other potential target antigens, including, but not limited to, prostate cancer specific antigen, hepatitis C antigen, Epstein-Barr virus antigen, HIV-1 and HIV-2 antigens, and papilloma virus antigens. As set forth in more detail below, usually at least four, sometimes six, often seven or more residues of the peptide or a majority of amino acids of that peptide will be identical or homologous when compared to the corresponding portion of the naturally occurring HBenv sequence identified as HBenv₃₀₉₋₃₂₈ (peptide 799.08) or HBenv₃₂₉₋₃₄₈ (peptide 799.09) or HBenv₃₄₉₋₃₆₈ (peptide 799.10), or the HBc region HBc₉₁₋₁₁₀ (peptide 802.03).

The peptides can be prepared "synthetically," as described hereinbelow, or by recombinant DNA technology. Although the peptide will preferably be substantially free of other naturally occurring HBV proteins and fragments thereof, in some embodiments the peptides can be synthetically conjugated to native fragments or particles. The term peptide is used interchangeably with polypeptide in the present specification to designate a series of amino acids connected one to the other by peptide bonds between the alpha-amino and alpha-carboxy groups of adjacent amino acids. The polypeptides or peptides can be a variety of lengths, either in their neutral (uncharged) forms or in forms which are salts, and either free of modifications such as glycosylation, side chain oxidation, or phosphorylation or containing these modifications, subject to the condition that the modification not destroy the biological activity of the polypeptides as herein described.

Desirably, the peptide will be as small as possible while still maintaining substantially all of the biological activity of the large peptide. When possible, it may be desirable to optimize peptides of the invention to a length of eight to twelve amino acid residues, commensurate in size with endogenously processed viral peptides that are bound to MHC class I molecules on the cell surface. See generally, Schumacher et al., *Nature* 350:703-706 (1991); Van Bleek et al., *Nature* 348:213-216 (1990); Rotzschke et al., *Nature* 348:252-254 (1990); and

Falk et al., *Natur* 351:290-296 (1991), which are incorporated herein by reference. By biological activity is meant the ability to bind an appropriate MHC molecule and, in the case of peptides useful for stimulating CTL responses, induce a CTL response against HBV antigen or antigen mimetic. In the case of a peptide analog antagonist, the analog will have biological activity if it competes with the peptide for binding to the MHC molecule and has a substantially reduced ability to stimulate a CTL response when compared to the native peptide. By a CTL response is meant a CD8⁺ T lymphocyte response specific for an HBV antigen of interest, wherein CD8⁺, MHC class I-restricted T lymphocytes are activated. As noted above, the activated T lymphocytes will secrete a variety of products which inhibit viral replication and may or may not kill the HBV infected (or transfected) cell.

The terms "homologous", "substantially homologous", and "substantial homology" as used herein denote a sequence of amino acids having at least 50% identity wherein one sequence is compared to a reference sequence of amino acids. The percentage of sequence identity or homology is calculated by comparing one to another when aligned to corresponding portions of the reference sequence.

A CTL inducing HBenv peptide embodiment of the invention comprises from six to thirty amino acids and is derived from the 799.08 peptide region HBenv₃₀₉₋₃₂₈, contains a CTL epitopic site(s), and has at least seven amino acids wherein a majority of amino acids of the peptide will be identical or substantially homologous, when compared to the amino acids comprising the corresponding portion of the naturally occurring HBenv₃₀₉₋₃₂₈ sequence. A representative peptide of this region is peptide HBenv₃₀₉₋₃₂₈, which has the following sequence (for HBV subtype ayw):

799.08 (HBenv₃₀₉₋₃₂₈) [Seq. ID No. 1]

Asn-Cys-Thr-Cys-Ile-Pro-Ile-Pro-Ser-Ser-Trp-Ala-
Phe-Gly-Lys-Phe-Leu-Trp-Glu-Trp

wherein the peptide can be optionally flanked and/or modified at one or both of the N- and C-termini, as desired, by amino acids from HBV sequences, particularly HBenv, amino acids added to facilitate linking, other N- and C-terminal modifications, linked to carriers, etc., as further described herein. For a peptide of the HBV subtype adw, Gly₃₂₂ is replaced by Ala, and Phe₃₂₄ is replaced by Tyr. The peptide HBenv₃₀₉₋₃₂₈ induces a CTL response which is mediated by at least the MHC class I molecule HLA-A2.

Another HBenv CTL inducing peptide embodiment of the invention comprises from six to twenty amino acids of the 799.10 peptide region HBenv₃₄₉₋₃₆₈, and includes peptides derived from HBenv₃₄₉₋₃₆₈ which contain an epitopic site(s) of at least seven or more amino acids where a majority of amino acids of the peptide will be identical or homologous when compared to the corresponding portion of the naturally occurring HBenv sequence identified as HBenv₃₄₉₋₃₆₈, which is as follows (for HBV subtypes ayw and adw):

799.10 (HBenv₃₄₉₋₃₆₈) [Seq. ID No. 2]

Leu-Ser-Pro-Thr-Val-Trp-Leu-Ser-Val-Ile-
Trp-Met-Met-Trp-Tyr-Trp-Gly-Pro-Ser-Leu

wherein the peptide selected from said region can be flanked and/or modified at one or both termini as described herein. An example of another CTL inducing peptide derived from the region of 799.10 (HBenv₃₄₉₋₃₆₈) [Seq. ID No. 2] which contains at least one epitope capable of inducing a MHC class I-restricted cytotoxic T-lymphocyte response to hepatitis B virus is:

884.02 (HBenv₃₄₉₋₃₅₈) [Seq. ID No. 3]

Leu-Ser-Pro-Thr-Val-Trp-Leu-Ser-Val-Ile.

Yet other CTL-inducing peptide embodiments of the invention are derived from the HBenv region HBenv₃₂₉₋₃₄₈. In addition to peptide 799.09 (HBenv₃₂₉₋₃₄₈), these embodiments include peptides which contain an epitopic site(s) within the sequence of HBenv₃₂₉₋₃₄₈ which is capable of inducing a MHC class I-restricted CTL response to HBV. HBenv₃₂₉₋₃₄₈ for HBV subtype ayw has the following sequence:

799.09 (HBenv₃₂₉₋₃₄₈) [Seq. ID No. 7]
 Ala-Ser-Ala-Arg-Phe-Ser-Trp-Leu-Ser-Leu-
 Leu-Val-Pro-Phe-Val-Gln-Trp-Phe-Val-Gly.

In further embodiments, peptides of the invention are derived from the HBc region HBc₉₁₋₁₁₀, and besides peptide 802.03 (HBc₉₁₋₁₁₀) includes peptides which contain an epitopic site(s) of at least seven and preferably nine amino acids wherein a majority of amino acids of the peptide will be identical or substantially homologous when compared to the amino acids of the corresponding portion of the naturally occurring HBc sequence of HBc₉₁₋₁₁₀, wherein the sequence for HBc₉₁₋₁₁₀ for HBV subtype ayw has the following sequence:

802.03 (HBc₉₁₋₁₁₀) [Seq. ID No. 4]
 Thr-Asn-Met-Gly-Leu-Lys-Phe-Arg-Gln-Leu-
 Leu-Trp-Phe-His-Ile-Ser-Cys-Leu-Thr-Phe

wherein the peptide selected from said region can be flanked and/or modified at one or both termini as described herein. For a peptide of the HBV subtype adw, Phe₉₇ is replaced by Ile, and Leu₁₀₁ is replaced by Trp. The CTL inducing peptide 802.03 induces a CTL response which is mediated by at least the MHC class I molecule HLA-A2.1. Examples of CTL inducing peptides derived from the region of 802.03 (HBc₉₁₋₁₁₀) [Seq. ID No. 4] and which contain an epitope capable of inducing a MHC class I-restricted cytotoxic T-lymphocyte response to hepatitis B virus include the following:

883.02 (HBc₉₂₋₁₀₁) [Seq. ID No.]
 Asn-Met-Gly-Leu-Lys-Phe-Arg-Gln-Leu-Leu,

(HBc₉₂₋₁₀₀) [Seq. ID No.]
 Asn-Met-Gly-Leu-Lys-Phe-Arg-Gln-Leu, and

883.03 (HBc₉₃₋₁₀₂) [Seq. ID No. 6]
 Met-Gly-Leu-Lys-Phe-Arg-Gln-Leu-Leu-Trp.

As mentioned above, additional amino acids can be added to the termini of an oligopeptide or peptide to provide for ease of linking peptides one to another, for coupling to a carrier, support or larger peptide, for reasons discussed herein, for modifying the physical or chemical properties of the peptide or oligopeptide, or the like. Amino acids such as tyrosine, cysteine, lysine, glutamic or aspartic acid, or the like, can be introduced at the C- or N-terminus of the peptide or oligopeptide. In addition, the peptide or oligopeptide sequences can differ from the natural sequence by being modified by terminal-NH₂ acylation, e.g., by alkanoyl (C₁-C₂₀) or thio-glycolyl acetylation, terminal-carboxy amidation, e.g., ammonia, methylamine, etc. In some instances these modifications may provide sites for linking to a support or other molecule.

It will be understood that the peptides of the present invention or analogs thereof which have CTL stimulating activity may be modified to provide other desired attributes, e.g., improved pharmacological characteristics, while increasing or at least retaining substantially all of the biological activity of the unmodified peptide. For instance, the peptides can be modified by extending, decreasing or substituting in the peptide's amino acid sequences by, e.g., the addition or deletion of amino acids on either the amino terminal or carboxy terminal end, or both, of peptides derived from the sequences disclosed herein. As further described below, the CTL activity of the subject peptides can be enhanced by linkage to a sequence which contains at least one epitope that is capable of inducing a T helper cell response, such as contained within a T helper peptide provided by peptides from tetanus toxoid 830-843, influenza 307-319, malaria circumsporozoite 382-398 and 378-389,

ovalbumin 323-336, and HBc128-140, HBc1-20, HBc50-69 and HBc111-125.

The peptides employed in the subject invention need not be identical to peptides 799.08, 799.09, 799.10 or 802.03, or to a particular HBV surface antigen or nucleocapsid protein sequence, so long as the subject compounds are able to bind to the appropriate MHC molecule and provide for cytotoxic T lymphocytic activity against at least one of the four major subtypes of HBV (except in the case of peptide analog antagonists, which bind the MHC molecule but have a substantially reduced ability to stimulate CTL activity, as explained further herein). Therefore, the peptides may be subject to various changes, such as insertions, deletions, and substitutions, either conservative or non-conservative, where such changes might provide for certain advantages in their use. By conservative substitutions is meant replacing an amino acid residue with another which is biologically and/or chemically similar, e.g., one hydrophobic residue for another, or one polar residue for another. The substitutions include combinations such as Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. Usually, the portion of the sequence which is intended to substantially mimic an HBV CTL stimulating epitope will not differ by more than about 20% from the sequence of at least one subtype of HBV, except where additional amino acids may be added at either terminus for the purpose of modifying the physical or chemical properties of the peptide for, e.g., ease of linking or coupling, and the like. In those situations where regions of the peptide sequences are found to be polymorphic among HBV subtypes, it may be desirable to vary one or more particular amino acids to more effectively mimic differing cytotoxic T-lymphocyte epitopes of different HBV strains or subtypes.

Within the peptide sequence regions identified by the present invention as containing CTL epitopes, e.g., peptide region 799.08 (HBenv₃₀₉₋₃₂₈), peptide region 799.09 (HBenv₃₂₉₋₃₄₈), peptide region 799.10 (HBenv₃₄₉₋₃₆₈), or peptide region 802.03 (HBc₉₁₋₁₁₀), there are residues (or those which are substantially functionally equivalent) which allow the peptide to retain their biological activity, i.e., the ability to stimulate a class I-restricted cytotoxic T-lymphocytic response against HBV infected cells or cells which express HBV surface and/or nucleocapsid antigens. These residues can be identified by single amino acid substitutions, deletions, or insertions. In addition, the contributions made by the side chains of the residues can be probed via a systematic scan with a specified amino acid (e.g., Ala). Peptides which tolerate multiple substitutions generally incorporate such substitutions as small, relatively neutral molecules, e.g., Ala, Gly, Pro, or similar residues. The number and types of residues which can be substituted, added or subtracted will depend on the spacing necessary between the essential epitopic points and certain conformational and functional attributes which are sought (e.g., hydrophobicity vs. hydrophilicity). If desired, increased binding affinity of peptide analogues to its MHC molecule for presentation to a CTL can also be achieved by such alterations. Generally, any spacer substitutions, additions or deletions between epitopic and/or conformationally important residues should employ amino acids or other moieties chosen to avoid steric and charge interference which might disrupt binding.

Peptides which tolerate substitutions while retaining the desired biological activity may also be synthesized as D-amino acid containing peptides. Such peptide may be synthesized as "inverso" or "retro-inverso" forms, that is, by replacing L-amino acids of a sequence with D-amino acids, or by reversing the sequence of the amino acids and replacing the L-amino acids with D-amino acids. As the D-peptides are substantially more resistant to peptidases, and therefore are more stable in serum and tissues compared to their L-peptide counterparts, the stability of D-peptides under physiological conditions may more than compensate for a difference in affinity compared to the corresponding L-peptide. Further, L-amino acid-containing peptides with or without substitutions can be capped with a D-amino acid to inhibit exopeptidase destruction of the antigenic peptide.

Having identified different peptides of the invention which contribute to stimulating HBV specific CTL responses in one or more patients or HLA types, in some instances it may be desirable to join two or more peptides in a composition. The peptides in the composition can be identical or different, and together they should provide equivalent or greater biological activity than the parent peptide(s). For example, using the methods described herein, two or more peptides may define different or overlapping CTL epitopes from a particular region, e.g., the peptide region 799.08 (HBenv₃₀₉₋₃₂₈), peptide region, 799.09 (HBenv₃₂₉₋₃₄₈), 799.10 (HBenv₃₄₉₋₃₆₈), or peptide region 802.03 (HBc₉₁₋₁₁₀), which peptides can be combined in a "cocktail" to provide enhanced immunogenicity for CTL responses. Peptides of one region can also be combined with peptides having different MHC restriction elements. This composition can be used to effectively broaden the immunological coverage provided by therapeutic, vaccine or diagnostic methods and compositions of the invention among a diverse population.

The peptides of the invention can be combined via linkage to form polymers (multimers), or can be formulated in a composition without linkage, as an admixture. Where the same peptide is linked to itself, thereby forming a homopolymer, a plurality of repeating epitopic units are present. When the peptides differ, e.g., a cocktail comprising different HBV subtypes, different epitopes within a subtype, different HLA restriction specificities, or peptides which contain different epitopes, heteropolymers with repeating units are provided. In

addition to covalent linkages, noncovalent linkages capable of forming intermolecular and intrastructural bonds are also contemplated.

Linkages for homo- or hetero-polymers or for coupling to carriers can be provided in a variety of ways. For example, cysteine residues can be added at both the amino- and carboxy-termini, where the peptides are covalently bonded via controlled oxidation of the cysteine residues. Also useful are a large number of hetero-bifunctional agents which generate a disulfide link at one functional group end and a peptide link at the other, including N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP). This reagent creates a disulfide linkage between itself and a cysteine residue in one protein and an amide linkage through the amino on a lysine or other free amino group in the other. A variety of such disulfide/amide forming agents are known. See, for example, *Immun. Rev.* 62:185 (1982). Other bifunctional coupling agents form a thioether rather than a disulfide linkage. Many of these thioether forming agents are commercially available and include reactive esters of 6-maleimidocaproic acid, 2 bromoacetic acid, 2-iodoacetic acid, 4-(N-maleimido-methyl) cyclohexane-1-carboxylic acid and the like. The carboxyl groups can be activated by combining them with succinimide or 1-hydroxy-2-nitro-4-sulfonic acid, sodium salt. A particularly preferred coupling agent is succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC). Of course, it will be understood that linkage should not substantially interfere with either of the linked groups to function as described, e.g., to function as an HBV cytotoxic T cell determinant, peptide analog CTL antagonist, or HBV T helper determinant.

In another aspect the peptides of the invention can be combined or coupled with other peptides which present HBV T-helper cell epitopes, i.e., T helper peptides comprising six to thirty amino acids containing a T helper epitope which from the envelope, core or other immunogenic protein or derivative thereof, stimulate T cells that cooperate in the induction of cytotoxic T cells to HBV. The T-helper cells can be either the T-helper 1 or T-helper 2 phenotype, for example. Compositions of T-helper peptides and CTL peptides thereby enhance an individual's immunity by providing cell-mediated immunity and protective antibodies to HBV. T-helper epitopes have been identified as HBC₁₋₂₀, having the sequence: Met-Asp-Ile-Asp-Pro-Tyr-Lys-Glu-Phe-Gly-Ala-Thr-Val-Glu-Leu-Leu-Ser-Phe-Leu-Pro. Other T-helper epitopes are provided by peptides from the region HBC₅₀₋₆₉, having the sequence Pro-His-His-Tyr-Ala-Leu-Arg-Gln-Ala-Ile-Leu-Cys-Trp-Gly-Glu-Leu-Met-Tyr-Leu-Ala, and from the region of HBC₁₀₀₋₁₃₉, including HBC₁₀₀₋₁₁₉ having the sequence Leu-Leu-Trp-Phe-His-Ile-Ser-Cys-Leu-Thr-Phe-Gly-Arg-Glu-Thr-Val-Ile-Glu-Tyr-Leu (where Ile₁₁₆ is Leu in the HBV adw subtype), HBC₁₁₇₋₁₃₁ having the sequence Glu-Tyr-Leu-Val-Ser-Phe-Gly-Val-Trp-Ile-Arg-Thr-Pro-Pro-Ala, and peptide HBC₁₂₀₋₁₃₉ having the sequence Val-Ser-Phe-Gly-Val-Trp-Ile-Arg-Thr-Pro-Pro-Ala-Tyr-Arg-Pro-PPro-Asn-Ala-Pro-Ile. See, Ferrari et al., *J. Clin. Invest.* 88:214-222 (1991), and U.S. Pat. No. 4,882,145, each of which is incorporated herein by reference. Other T helper epitopes are provided by peptides from, for example, tetanus toxoid₈₃₀₋₈₄₃ having the sequence Gln-Tyr-Ile-Lys-Ala-Asn-Ser-Lys-Phe-Ile-Gly-Ile-Thr-Glu; malaria circumsporozoite₃₈₂₋₃₉₈ Lys-Ile-Ala-Lys-Met-Lys-Ala-Ser-Ser-Val-Phe-Asn-Val-Val-Asn-Ser (KIAKMEKASSVFNVNS); malaria circumsporozoite₃₇₈₋₃₉₈ Asp-Ile-Glu-Lys-Lys-Ile-Ala-Lys-Met-Lys-Ala-Ser-Ser-Val-Phe-Asn-Val-Val-Asn-Ser (DIEKKIAKMEKASSVFNVNS); ovalbumin₃₂₃₋₃₃₆ Ile-Ser-Gln-Ala-Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-Glu and the influenza epitope₃₀₇₋₃₁₉ Pro-Lys-Tyr-Val-Lys-Gln-Asn-Thr-Leu-Lys-Leu-Ala-Thr.

In preferred embodiments the CTL inducing peptides of the invention are covalently linked to the T helper peptides. Particularly preferred CTL inducing peptides / T helper conjugates are linked by a spacer molecule. Alternatively, the CTL peptide may be linked to the T helper peptide without a spacer. The spacer is typically comprised of relatively small, neutral molecules, such as amino acids or amino acid mimetics, which are substantially uncharged under physiological conditions and may have linear or branched side chains. The spacers are typically selected from, e.g., Ala, Gly, or other neutral spacers of nonpolar amino acids or neutral polar amino acids. In certain preferred embodiments herein the neutral spacer is Ala. It will be understood that the optionally present spacer need not be comprised of the same residues and thus may be a hetero- or homo-oligomer. Preferred exemplary spacers are homo-oligomers of Ala. When present, the spacer will usually be at least one or two residues, more usually three to six residues. In other embodiments the T helper peptide is conjugated to the CTL peptide, preferably with the T helper peptide positioned at the amino terminus. The peptides may be joined by a neutral linker, such as Ala-Ala-Ala or the like, and preferably further contains a lipid residue such as palmitic acid, (PAM)₂ or the like (as described further below) which is attached to alpha and epsilon amino groups of a Lys residue, which is attached to the amino terminus of the peptide conjugate, typically via Ser-Ser linkage or the like.

The CTL inducing peptide may be linked to the T helper peptide either directly or via a spacer either at the amino or carboxy terminus of the CTL peptide. The amino terminus of either the CTL inducing peptide or the T helper peptide may be acylated. In addition, the CTL peptide/T helper conjugate may be linked to certain alkanoyl (C₁-C₂₀) lipids via one or more linking residues such as Gly, Gly-Gly, Ser, Ser-Ser as described below.

In an exemplary embodiment described below, a T helper peptide from substantially within HBC₁₂₈₋₁₄₀ (Thr-

Pro-Pro-Ala-Tyr-Arg-Pro-Pro-Asn-Ala-Pro-Ile-Leu) (Seq. ID No. [19]), when linked with a CTL peptide (HBc18-27), was shown to induce specific CTL priming of animals in all animals studied, and at levels which were greater than when the CTL peptide and T helper peptide were administered unlinked. When the T helper and CTL HBV peptides were linked by a Ala-Ala-Ala spacer, specific CTL activity greater than induction of specific CTL activity with the linked peptides without spacer. These results suggest enhanced CTL response against cells which display HBV antigen: when the peptide containing a CTL epitope is linked via spacer to a peptide containing a HBV T helper epitope is used as the immunogen.

The peptides of the invention can be prepared in a wide variety of ways. Because of their relatively short size, the peptides can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, Stewart and Young, Solid Phase Peptide Synthesis, 2d. ed., Pierce Chemical Co. (1984); Tam et al., J. Am. Chem. Soc. 105:6442 (1983); Merrifield, Science 232:341-347 (1986); and Barany and Merrifield, The Peptides, Gross and Meienhofer, eds., Academic Press, New York, pp. 1-284 (1979), each of which is incorporated herein by reference.

Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes a CTL peptide and/or T helper peptide of interest is inserted into an expression vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression. These procedures are generally known in the art, as described generally in Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, New York (1982), and Ausubel et al., (ed.) Current Protocols in Molecular Biology, John Wiley and Sons, Inc., New York (1987), and U.S. Pat. Nos. 4,237,224, 4,273,875, 4,431,739, 4,363,877 and 4,428,941, for example, which disclosures are incorporated herein by reference. Thus, fusion proteins which comprise one or more peptide sequences of the invention can be used to present the HBV cytotoxic T cell determinants. For example, a recombinant HBV surface antigen protein is prepared in which the HBenv amino acid sequence is altered so as to more effectively present epitopes of peptide regions described herein to stimulate a CTL response. By this means a polypeptide is used which incorporates several T cell epitopes.

As the coding sequence for peptides of the length contemplated herein can be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci et al., J. Am. Chem. Soc. 103:3185 (1981), modification can be made simply by substituting the appropriate base(s) for those encoding the native peptide sequence. The coding sequence can then be provided with appropriate linkers and ligated into expression vectors commonly available in the art, and the vectors used to transform suitable hosts to produce the desired fusion protein. A number of such vectors and suitable host systems are now available. For expression of the fusion proteins, the coding sequence will be provided with operably linked start and stop codons, promoter and terminator regions and usually a replication system to provide an expression vector for expression in the desired cellular host. For example, promoter sequences compatible with bacterial hosts are provided in plasmids containing convenient restriction sites for insertion of the desired coding sequence. The resulting expression vectors are transformed into suitable bacterial hosts. Of course, yeast or mammalian cell hosts may also be used, employing suitable vectors and control sequences.

The peptides of the present invention and pharmaceutical and vaccine compositions thereof are useful for administration to mammals, particularly humans, to treat and/or prevent HBV infection. As the peptides are used to stimulate cytotoxic T-lymphocyte responses to HBV infected cells, the compositions can be used to treat or prevent acute and/or chronic HBV infection.

For pharmaceutical compositions, the peptides, i.e., the CTL peptide or CTL/T helper peptide, of the invention as described above will be administered to an individual already infected with HBV. Those in the incubation phase or the acute phase of infection can be treated with the immunogenic peptides separately or in conjunction with other treatments, as appropriate. In therapeutic applications, compositions are administered to a patient in an amount sufficient to elicit an effective CTL response to HBV and to cure or at least partially arrest its symptoms and/or complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on, e.g., the peptide composition, the manner of administration, the stage and severity of the disease being treated, the weight and general state of health of the patient, and the judgment of the prescribing physician, but generally range for the initial immunization (that is for therapeutic or prophylactic administration) from about 1.0 µg to about 500 µg of peptide for a 70 kg patient, followed by boosting dosages of from about 1.0 µg to about 100 µg of peptide pursuant to a boosting regimen over weeks to months depending upon the patient's response and condition by measuring specific CTL activity in the patient's blood. It must be kept in mind that the peptides and compositions of the present invention may generally be employed in serious disease states, that is, life-threatening or potentially life threatening situations. In such cases, in view of the minimization of extraneous substances and the relative nontoxic nature of the peptides, it is possible and may be felt desirable by the treating physician to administer

substantial excesses of these peptide compositions.

Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of cytotoxic T-lymphocyte stimulatory peptides of the invention sufficient to effectively treat the patient.

For therapeutic use, administration should begin at the first sign of HBV infection or shortly after diagnosis in the case of acute infection, to be followed by boosting doses until at least symptoms are substantially abated and for a period thereafter. In chronic infection, loading doses followed by boosting doses may be required. The elicitation of an effective CTL response to HBV during treatment of acute hepatitis will minimize the possibility of subsequent development of chronic hepatitis, HBV carrier stage, and ensuing hepatocellular carcinoma.

Treatment of an infected individual with the compositions of the invention may hasten resolution of the infection in acutely infected individuals. For those individuals susceptible (or predisposed) to developing chronic infection the compositions are particularly useful in methods for preventing the evolution from acute to chronic infection. Where the susceptible individuals are identified prior to or during infection, for instance, as described herein, the composition can be targeted to them, minimizing need for administration to a larger population.

The peptide compositions can also be used for the treatment of chronic hepatitis and to stimulate the immune system of carriers to eliminate virus-infected cells. Those with chronic hepatitis can be identified as testing positive for virus from about 3-6 months after infection. As individuals may develop chronic HBV infection because of an inadequate (or absent) CTL response during the acute phase of their infection, it is important to provide an amount of immuno-potentiating peptide in a formulation and mode of administration sufficient to effectively stimulate a cytotoxic T cell response. Thus, for treatment of chronic hepatitis, a representative dose is in the range of about 1.0 µg to about 500 µg, preferably about 5 µg to 100 µg for a 70 kg patient per dose. Administration should continue until at least clinical symptoms or laboratory indicators indicate that the HBV infection has been eliminated or substantially abated and for a period thereafter. Immunizing doses followed by boosting doses at established intervals, e.g., from one to four weeks, may be required, possibly for a prolonged period of time, as necessary to resolve the infection. For the treatment of chronic and carrier HBV infection it may also be desirable to combine the CTL peptides with other peptides or proteins that induce immune response to other HBV antigens, such as HBsAg.

In another embodiment of the invention, chronic active hepatitis is treated using antigenic epitope analogs as antagonists. The analog antagonists bind to the appropriate MHC class I antigen, but prevent CTL activation and proliferation, thereby reducing damage to HBV infected hepatocytes and T cell mediated liver inflammation. The peptide antagonists comprise a CTL epitope peptide which has been modified, e.g., by amino acid substitution, such that the peptide binds effectively to the Class I MHC molecule but its ability to stimulate epitope specific T cells is substantially reduced.

Analog antagonists are identified by making modifications to a peptide which contains at least one HLA class I epitope. In preferred embodiments the peptide to be modified will comprise a class I-restricted CTL epitope. Substitutions or other selected modifications are introduced into the peptide, which peptide analog is then screened for the ability to block the presentation of an unrelated antigen epitope, such as an influenza peptide, to an influenza-specific T cell clone restricted by the same class I molecule. For example, an HLA-A2 restricted CTL peptide as described herein is modified, e.g., by one or more point substitutions, and then tested for the ability to block the presentation of HLA-A2-restricted influenza CTL matrix peptide (e.g., 56-68) to an influenza-specific T cell clone restricted by HLA-A2. Antagonist analogs will inhibit T cell proliferation and/or cytolytic activity when the antigen presenting cell/target cell is exposed to the antagonist peptide after exposure to a stimulatory dose of the antigenic peptide. Antagonist analogs of the invention can be identified for a variety of HBV class I epitopes, including those described in, e.g., U.S. Pat. No. 4,882,145 and Ferrari et al., *J. Clin. Invest.*, *supra*.

The pharmaceutical compositions for therapeutic treatment are intended for parenteral, topical, oral or local administration. Preferably, the pharmaceutical compositions are administered parenterally, e.g., intravenously, subcutaneously, intradermally, or intramuscularly. Thus, the invention provides compositions for parenteral administration which comprise a solution of the CTL stimulatory peptides dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium

chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

In some embodiments it may be desirable to include in the pharmaceutical composition at least one component which primes CTL. Lipids have been identified as agents capable of priming CTL *in vivo* against viral antigens. For example, palmitic acid residues can be attached to the alpha and epsilon amino groups of a Lys residue and then linked, e.g., typically via one or more linking residues such as Gly, Gly-Gly-, Ser, Ser-Ser, or the like, to a synthetic peptide which comprises a class I-restricted CTL epitope. As further described herein, the lipidated peptide can then be incorporated into a liposome emulsified in an adjuvant, e.g., incomplete Freund's adjuvant. In a preferred embodiment a particularly effective immunogen comprises palmitic acid attached to alpha and epsilon amino groups of Lys, which is attached via linkage, e.g., Ser-Ser, to the amino terminus of a class I restricted peptide having T cell determinants, such as those peptides described herein as well as other peptides which have been identified as having such determinants.

As another example of lipid priming of CTL responses, *E. coli* lipoprotein, such as tripalmitoyl-S-glyceryl-cysteinyl-seryl-serine (P₃CSS), can be used to prime virus specific CTL when covalently attached to an appropriate peptide. See, Deres et al., *Nature* 342:561-564 (1989), incorporated herein by reference. Peptides of the invention can be coupled to P₃CSS, for example, and the lipopeptide administered to an individual to specifically prime a CTL response to HBV. Further, as the induction of neutralizing antibodies can also be primed with P₃CSS conjugated to a peptide which displays an appropriate epitope, e.g., HBsAg epitopes, the two compositions can be combined to more effectively elicit both humoral and cell-mediated responses to HBV infection. Yet another example of lipid priming of CTL response is achieved by conjugating the CTL/T helper-peptide-conjugate with uncharged fatty acid residues of different chain lengths, ranging from acetic to stearic acid as well as to negatively charged succinyl residues via the appropriate carboxylic acid anhydrides.

CTL responses to a variety of other antigens may be enhanced by combining a CTL inducing peptide/T helper inducing peptide conjugate with a lipid. The CTL inducing peptides may be selected from target proteins, such as, e.g. prostate cancer specific antigen (PSA), hepatitis C antigens Epstein-Barr virus antigen, HIV-1 and HIV-2 antigens, and papilloma virus antigens, among others. The lipid may be linked to other peptides which present T helper epitopes which are then combined with the lipid. The arrangement of the components of the conjugate comprising the CTL inducing peptide/T helper peptide/lipid can be varied. In one case, the lipid moiety can be linked to the amino terminal end of the CTL inducing peptide, which is then linked at its carboxy terminal to the T helper peptide. In another case, the lipid is linked at the amino terminal end of the T helper peptide, which is linked at its carboxy terminal to the CTL inducing peptide. In each case, a spacer molecule can be selectively inserted between the lipid moiety and the CTL or T helper peptide, as well as between the T helper and the CTL inducing peptides. In the case of the spacer between the lipid and the T helper or CTL inducing peptide, and a preferred example is comprised of Lys-Ser-Ser, although other spacers are described herein. An example of a spacer between the T helper and CTL inducing peptides will be Ala-Ala-Ala, as also described in further detail herein. The CTL inducing peptide can be from the HBc or HBs region, or from other CTL inducing antigens as noted above.

The concentration of CTL stimulatory peptides of the invention in the pharmaceutical formulations can vary widely, i.e., from less than about 1%, usually at or at least about 10% to as much as 20 to 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

Thus, a typical pharmaceutical composition for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 100 mg of peptide. Actual methods for preparing parenterally administrable compounds will be known or apparent to those skilled in the art and are described in more detail in for example, *Remington's Pharmaceutical Sciences*, 17th ed., Mack Publishing Company, Easton, PA (1985), which is incorporated herein by reference.

The peptides of the invention may also be administered via liposomes, which serve to target the peptides to a particular tissue, such as lymphoid tissue, or targeted selectively to HBV infected hepatic cells, as well as increase the half-life of the peptide composition. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations the peptide to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to, e.g., a receptor prevalent among lymphoid cells, such as monoclonal antibodies which bind to the CD45 antigen, or with other therapeutic or immunogenic compositions. Thus, liposomes filled with a desired peptide of the invention can be directed to the site of lymphoid cells, where the liposomes then deliver the selected therapeutic/immunogenic peptide compositions. Liposomes for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka et al., *Ann. Rev. Biophys. Bioeng.* 9:467 (1980), U.S. Patent Nos. 4,235,871,

4,501,728, 4,837,028, and 5,019,369, incorporated herein by reference. For targeting to the immune cells, a ligand to be incorporated into the liposome can include, e.g., antibodies or fragments thereof specific for cell surface determinants of the desired immune system cells. A liposome suspension containing a peptide may be administered intravenously, locally, topically, etc. in a dose which varies according to, inter alia, the manner of administration, the peptide being delivered, and the stage of the disease being treated.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more peptides of the invention, and more preferably at a concentration of 25%-75%.

For aerosol administration, the CTL stimulatory peptides are preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of peptides are 0.01%-20% by weight, preferably 1%-10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1%-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery.

In another aspect the present invention is directed to vaccines which contain as an active ingredient an immunogenically effective amount of a CTL stimulating peptide as described herein. The peptide(s) may be introduced into a host, including humans, linked to its own carrier or as a homopolymer or heteropolymer of active peptide units. Such a polymer has the advantage of increased immunological reaction and, where different peptides are used to make up the polymer, the additional ability to induce antibodies and/or cytotoxic T cells that react with different antigenic determinants of HBV. Useful carriers are well known in the art, and include, e.g., thyroglobulin, albumins such as human serum albumin, tetanus toxoid, polyamino acids such as poly(D-lysine:D-glutamic acid), influenza, hepatitis B virus core protein, hepatitis B virus recombinant vaccine and the like. The vaccines can also contain a physiologically tolerable (acceptable) diluent such as water, phosphate buffered saline, or saline, and further typically include an adjuvant. Adjuvants such as incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum are materials well known in the art. And, as mentioned above, CTL responses can be primed by conjugating peptides of the invention to lipids, such as P₃CSS. Upon immunization with a peptide composition as described herein, via injection, aerosol, oral, transdermal or other route, the immune system of the host responds to the vaccine by producing large amounts of CTLs specific for HBV surface and/or nucleocapsid antigen, and the host becomes at least partially immune to HBV infection, or resistant to developing chronic HBV infection.

Vaccine compositions containing the peptides of the invention are administered to a patient susceptible to or otherwise at risk of HBV infection to enhance the patient's own immune response capabilities. Such an amount is defined to be a "immunogenically effective dose." In this use, the precise amounts again depend on the patient's state of health and weight, the mode of administration, the nature of the formulation, etc., but generally range from about 1.0 µg to about 500 µg per 70 kilogram patient, more commonly from about 50 µg to about 100 µg mg per 70 kg of body weight. The peptides are administered to individuals of an appropriate HLA type, e.g., for vaccine compositions of peptide HBenv₃₀₉₋₃₂₈, HBenv₃₄₉₋₃₆₈ and HBc₉₁₋₁₁₀, these will be administered to HLA-A2 individuals.

In some instances it may be desirable to combine the peptide vaccines of the invention with vaccines which induce neutralizing antibody responses to HBV, particularly to HBV envelope antigens, such as recombinant HBV env-encoded antigens or vaccines prepared from purified plasma preparations obtained from HBV-infected individuals. A variety of HBV vaccine preparations have been described, and are based primarily on HBsAg and polypeptide fragments thereof. For examples of vaccines which can be formulated with the peptides of the present invention, see generally, European Patent publications EP 154,902 and EP 291,586, and U.S. Patent Nos. 4,565,697, 4,624,918, 4,599,230, 4,599,231, 4,803,164, 4,882,145, 4,977,092, 5,017,558 and 5,019,386, each of which is incorporated herein by reference. The vaccines can be combined and administered concurrently, or as separate preparations.

For therapeutic or immunization purposes, the peptides of the invention can also be expressed by attenuated viral hosts, such as vaccinia or fowlpox. This approach involves the use of vaccinia virus as a vector to express nucleotide sequences that encode the peptides of the invention. Upon introduction into an acutely or chronically HBV-infected host or into a non-infected host, the recombinant vaccinia virus expresses the HBs

and/or HBc peptide, and thereby elicits a host CTL response to HBV. Vaccinia vectors and methods useful in immunization protocols are described in, e.g., U.S. Patent No. 4,722,848, incorporated herein by reference. Another vector is BCG (bacille Calmette Guérin). BCG vectors are described in Stover et al. (*Nature* 351:456-460 (1991)) which is incorporated herein by reference. A wide variety of other vectors useful for therapeutic administration or immunization of the peptides of the invention, e.g., *Salmonella typhi* vectors and the like, will be apparent to those skilled in the art from the description herein.

The peptides may also find use as diagnostic reagents. For example, a peptide of the invention may be used to determine the susceptibility of a particular individual to a treatment regimen which employs the peptide or related peptides, and thus may be helpful in modifying an existing treatment protocol or in determining a prognosis for an affected individual. In addition, the peptides may also be used to predict which individuals will be at substantial risk for developing chronic HBV infection.

The following examples are offered by way of illustration, not by way of limitation.

EXAMPLE I

Identification of CTL-Specific HBV Epitopes

A line of transgenic mice which express a mouse-human chimeric class I molecule was used to define HBV core and surface antigen sequences that represent CTL-specific epitopes.

The transgenic mouse line 66 obtained from Scripps Clinic and Research Foundation expresses a chimeric class I molecule composed of the $\alpha 1$ and $\alpha 2$ domains of human HLA-A2.1 antigen and the $\alpha 3$ transmembrane and cytoplasmic domains of H-2K^b. The transgenic mice were prepared as generally described in Vitiello et al., *J. Exp. Med.* 173:1007-1015 (1991), which is incorporated herein by reference. When these mice are primed *in vivo* with the influenza virus, they generate a CTL response that is specific for virtually the same epitopes as those recognized by human influenza-specific CTL. Thus, these transgenic animals can be used to determine HBV epitopes recognized by human T cells.

To define which sequence regions within HBV surface and core proteins represented CTL epitopes, synthetic peptides derived from the two proteins were prepared and tested for their ability to bind to human HLA-A2.1. Binding was determined by the relative capacity of different peptide concentrations to inhibit recognition of A2.1 target cells in the presence of the influenza matrix peptide 57-68 by the CTL line 219, as determined by the inhibition of release of serine esterase from the cells. The 219 line was derived from A2.1 transgenic mice and is specific for the matrix peptide 57-68 in the context of HLA-A2.1.

Briefly, peptides to be assayed for CTL epitopes were dissolved in DMSO at a concentration of 20 mg/ml. Just before the assay, peptides were diluted in RPMI 1640 buffered with 25 μ M Hepes and containing .05% BSA (assay media). Fifty microliters of a 200 μ g/ml, 66 μ g/ml, or 22 μ g/ml of peptide solution were added to wells of 96 round-bottomed plates containing 4×10^5 Jurkat A2.1/K^b cells in a volume of 50 μ l of assay media. Plates were incubated for 30 min. at 37°C. Fifty μ l of 2.5 μ g/ml solution of the index peptide (matrix peptide 57-68 from PR8 influenza virus) were then added to the cells, followed by 50 μ l containing 5×10^4 line 219 CTL, where the concentration of index peptide used was chosen as that which induced 75% serine esterase release from CTL 219, as determined by titration of the peptide. After 4 hours incubation at 37°C, plates were centrifuged for 5 min. at 1000 RPM, and 20 μ l supernatant transferred to flat-bottomed 96-well plates. Esterase activity in the supernatant was measured by adding 180 μ l of a reaction mixture consisting of 0.2M TrisHCl pH 8.1, 2.0×10^{-4} N-benzyloxycarbonyl-L-Lysine thiobenzyl ester (BLT) and 2.2×10^{-4} M dithiobis (nitrobenzoic acid). Plates were incubated for 1 hour at 37°C and absorbance read at 412 nm. Percent inhibition was calculated by the following formula:

$$\% \text{ inhibition} = 100 - \frac{A_{412}(\text{test} + \text{index}) \text{ peptide} - A_{412} \text{ test peptide alone}}{A_{412} \text{ index peptide} - A_{412} \text{ no peptide}} \times 100$$

Those peptides which bound to A2.1 and caused more than 25% inhibition of serine esterase release by the cells were assayed *in vitro* for the ability to restimulate a CTL response from splenocytes derived from HBV primed A2.1 transgenic mice. HBV priming was performed by injecting A2.1 spleen cells "loaded" with HBV virus as described by Carbone and Bevan, *J. Exp. Med.* 171:377-387 (1990). Briefly, red blood cell depleted splenocytes were suspended in .4 ml of a solution composed of 200 μ l of HBV purified virus and 200 μ l of a 2 x hypertonic solution (0.5 M sucrose, 10% w/v polyethylene glycol 1000, 10 mM Hepes, pH 7.2, in RPMI 1640 medium), for 10 min. at 37°C. The cell suspension was then rapidly diluted in prewarmed hypotonic media (60% HBSS and 40% water), incubated for 2 min. at 37°C, pelleted, washed twice in HBSS and irradiated (1,000 rad.). Mice were then injected with 5.0×10^6 loaded cells in a volume of 200 μ l. Mice were boosted with HBV-loaded spleen cells 10 days later.

After about 2 weeks, spleen cells from primed mice (5×10^6 cells/well in 24 well plates) were cultured with 4 different mixtures of syngeneic irradiated (3000 rads) LPS blasts (2×10^6 cells/well) that had been independently coated with 13 different peptides. Coating was achieved by incubating aliquots of 25×10^6 LPS blasts in tubes each with 100 μ g of one of the 13 HBV synthetic peptides in one mL for 1-2 hrs at 37°C; the contents of the different tubes were then pooled to give 4 mixtures.

Mixture No.	Peptide No.	Peptide Location
1	800.04	HBenv47-63
	802.01	HBc11-27
	802.06	HBc162-176
2	801.02	HBenv141-157
	799.02	HBenv194-213
	802.03	HBc91-110
3	799.09	HBenv329-348
	799.10	HBenv349-368
	802.04	HBc111-125
4	799.04	HBenv234-253
	799.05	HBenv246-265
	799.08	HBenv309-328
	800.05	HBenv63-77

The mixture of cells was washed once, diluted at the required concentration and plated. The medium used for the cultures was RPMI 1640 supplemented with 10% FCS, 50 μ g/ml gentamicin, 2mM glutamine and 5×10^{-5} M 2-mercaptoethanol (R10). After nine days, effector cells were assayed for cytotoxicity against Jurkat A₂/k^b target cells in the presence of different peptide mixtures corresponding to those used in the cultures. The results obtained are shown in Fig. 1 panels, A through D. The effector cells (0.2×10^6 cells/well) obtained from these cultures were restimulated with irradiated (20,000 rads), peptide-coated Jurkat A₂/K^b cells ($.2 \times 10^6$ cells/well) in the presence of 3×10^6 feeder cells/well (C57BL/6 irradiated spleen cells) in R10 supplemented with 5%-rat ConA supernatant. After 6 days, these effector cells were assayed for cytotoxicity against ⁵¹Cr labeled Jurkat A₂/K^b target cells in the presence of the 13 individual peptides. Peptides that induced CTL lysis of Jurkat A₂/K^b target cells above background (Fig. 1, panels E through H) i.e., HBenv 47-63, HBc 11-27 (panel E) HBenv 141-157, HBenv 194-213, HBc 91-110 (panel F), HBenv 329-348 and 349-368 (panel G) and HBenv 309-328 (panel H) were independently used to restimulate the effector cells generated with the peptide mixtures. After 6d in culture, the effector cells were tested for cytotoxicity against ⁵¹Cr Jurkat A₂/K^b cells in the presence of the peptide used for the restimulation (Fig. 1). The set of experiments, outlined in this example allow us to determine that HBV peptides HBc 11-27 (Fig. 1 panels A,E; Fig. 2 panel J) HBc 91-110 (Fig. 1 panels B,F; Fig. 2 panel M), HBenv 329-348 (Fig. 1 panels C,G; Fig. 2 panel N) HBenv 349-368 (Fig. 1 panels C,G; Fig. 2 panel O) and HBenv 309-328 (Fig. 1 panels D,H; Fig. 2 panel P) clearly represent CTL epitopes.

EXAMPLE II

Induction of A2.1-restricted CTL by Subcutaneous Priming with Purified HBV in Incomplete Freund's Adjuvant (IFA)

Injection of ovalbumin (OVA) in IFA subcutaneously induces an ovalbumin-specific CTL response in mice, while injection of OVA either i.v. or i.p. generally does not lead to the generation of CTL. This technique was used to induce HBV-specific CTL in A2.1 transgenic mice.

Priming and In Vitro Restimulation: A2.1/K^b transgenic mice were injected with 100 microliters of an emulsion of purified HBV virus in incomplete Freund's adjuvant (IFA). This emulsion was prepared by mixing purified HBV (1 mg protein/ml) diluted 1:5 in HBSS with an equal volume of IFA. Seven days after priming, splenocytes

(5×10^6 cells/well in a 24 well plate) obtained from these animals were restimulated with syngeneic irradiated LPS blasts (2×10^6 /well) coated with each of the following peptides:

5	799.09	HBenv 329-348	802.03	HBc 91-110
	875.20	HBenv 335-343	883.02	HBc 92-101
10	875.21	HBenv 338-347	883.03	HBc 93-102
	799.10	HBenv 349-368	875.15	HBc 18-27
	884.01	HBenv 348-357	875.18	HBc 107-115
	884.02	HBenv 349-358	875.19	HBc 139-148

15 These peptides were chosen because: 1) They had been defined as containing CTL epitopes in Example I (peptides 799.10, 799.09, 802.03); 2) they represent truncations of peptides defined in Example I that are recognized by the CTL raised against the larger epitopes (i.e., peptides 875.15, 884.02, 883.02, 883.03); or 3) they contain the A2.1 binding motif as described by Falk et al. (*Nature* 351:290-296 (1991)), i.e., leucine or methionine in position 2, and either leucine or valine in position 9 or valine in position 10, (i.e., peptides 884.01, 20 875.20, 875.21, 875.18 and 875.19). Coating was achieved by incubating 50 μ g of each individual peptide with 12×10^6 LPS blasts in a volume of 0.4 ml of RPMI medium supplemented with 10% FCS for 1h at 37°C. The cells were washed once. After 6 days, effector cells were assayed for cytotoxicity against ^{51}Cr labelled Jurkat A2/K^b cells in the presence of the appropriate peptides. The results are shown in Fig. 3.

25 These effector cells (2×10^6 cells/well) were restimulated at weekly intervals. For the first restimulation, peptide-coated LPS blasts were used, followed by peptide-coated Jurkat A2.1/K^b cells. Six days after restimulation, effector cells were assayed for cytotoxicity against ^{51}Cr labelled Jurkat A2/K^b target cells in the presence of the appropriate peptides. The results obtained are shown in Fig. 4.

Peptides clearly able to induce *in vitro* CTL from splenocytes of HBV-primed mice are Fig. 3 and 4, panel A: HBc 18-27; Fig. 3 and 4, panel B: HBenv 349-368; Fig. 3 and 4, panel D: HBenv 349-358; Fig. 3 and 4, 30 panel F: HBenv 329-348; Fig. 3 and 4, panel I: HBc 91-110; Fig. 3 and 4, panel J: HBc 92-102; and Fig. 3 and 4, panel K: HBc 93-102. Truncation peptides recognized by CTL raised against the larger peptide and as such should contain at least part of a CTL epitope are: Fig. 3F, 4F: HBenv 335-343 and HBenv 338-347.

EXAMPLE III

35 Synthesis of Peptides

Peptides were synthesized on an Applied Biosystems (Foster City, CA) 430A peptides synthesizer using Fmoc protected amino acids and 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) esters for amino acid activation. Each amino acid was routinely triple coupled. Fmoc protected amino acids and Hydroxybenzotriazole were purchased from Burdick and Jackson. HBTU was purchased from Richelieu Biotechnologies (St-Hyacinthe, Canada). Piperidine and trifluoroacetic acid, acetic anhydride, and ethanedithiol were purchased from Sigma Chemical Corporation.

45 a. Peptide Phe-Leu-Pro-Ser-Asp-Phe-Phe-Pro-Ser-Val-OH

L-Valine coupled to Sasrin® resin (Bachem Biosciences) was loaded into the peptide synthesis reaction vessel and washed one time with N-methylpyrrolidone (NMP). The following operations were then sequentially performed:

- 50 1. The Fmoc protecting group was removed by treatment of the resin bound amino acid with 25% piperidine in NMP.
2. The resin was washed 5 times with NMP.
3. A mixture containing Fmoc-serine, diisopropylethylamine, HBTU and NMP was added to the reaction vessel and allowed to react for 30 minutes, under vortex agitation.
- 55 4. The solvent was drained, and the resin was washed three times with NMP.
5. Steps (3) and (4) were repeated two more times.
6. The resin was washed four more times with NMP.

Steps 1-6 were repeated for each amino acid of the peptide. Following the final coupling cycle, the resin-bound

peptide was deprotected by reaction with 25% piperidine in NMP, washed 7 times with NMP, and washed 2 times with dichloromethane. The resin was dried in vacuo for 24 hours. The peptide was cleaved from the Sasrin® resin by treatment with trifluoroacetic acid containing 2.5% ethanedithiol and 5% water. The polystyrene resin was separated from the trifluoroacetic acid solution by filtration. Trifluoroacetic was removed by evaporation in vacuo. The crude peptide was triturated with diethylether and dissolved in water. The water was removed by lyophilization. The peptide was then purified by reverse phase HPLC on a C₈ column (VYDAC) using a gradient of acetonitrile, water, each containing 0.1% TFA as modifier.

b. Peptide (Pal)₂-Lys-Ser-Ser-Phe-Leu-Pro-Ser-Asp-Phe-Phe-Pro-Ser-Val-OH

The resin bound peptide described in section a was extended by the addition of two serine residues according to the above described procedure. The following operations were then performed:

1. The Fmoc protecting group was removed by treatment of the resin bound amino acid with 25% piperidine in NMP.
2. The resin was washed 5 times with NMP.
3. Bis-Fmoc-Lysine was converted to the corresponding symmetrical anhydride by treatment with diisopropylcarbodiimide in NMP. The resin bound peptide was allowed to react with the resulting anhydride.
4. The resin was washed 5 times with NMP.
5. The Fmoc protecting group was removed by treatment of the resin bound amino acid with 25% piperidine in NMP.
6. Palmitic acid was reacted with hydroxybenzotriazole and diisopropylcarbodiimide in NMP. The resin bound peptide was allowed to react with the resulting solution.
7. The resin was washed 5 times with NMP.

Finally, the peptide was cleaved from the resin as described above.

c. Peptide Gln-Tyr-Ile-Lys-Ala-Asn-Ser-Lys-Phe-Ile-Gly-Ile-Thr-Glu-Phe-leu-Pro-Ser-Asp-Phe-Phe-Pro-Ser-Val-OH.

The resin bound peptide described in section a was chain extended by the addition of Glu, Thr, Ile, Gly, Ile, Phe, Lys, Ser, Asn, Ala, Lys, Ile, Tyr, and Gln residues, according to the procedure described in Section a. Cleavage and purification were performed as described above.

d. Peptide Gln-Tyr-Ile-Lys-Ala-Asn-Ser-Lys-Phe-Ile-Gly-Ile-Thr-Glu-Ala-Ala-Ala-Phe-Leu-Pro-Ser-Asp-Phe-Phe-Pro-Ser-Val-OH

The resin bound peptide described in section a was chain extended by the sequential addition of Ala, Ala, Ala, Glu, Thr, Ile, Gly, Ile, Phe, Lys, Ser, Asn, Ala, Lys, Ile, Tyr, and Gln residues, according to the procedure described in section a. Cleavage and purification were performed as described above.

e. Peptide Ac-Gln-Tyr-Ile-Lys-Ala-Asn-Ser-Lys-Phe-Ile-Gly-Ole-Thr-Glu-Ala-Ala-Ala-Phe-Leu-Pro-Ser-Asp-Phe-Phe-Pro-Ser-Val-OH

The resin bound peptide described in Section d was acetylated by reaction with acetic anhydride in NMP. Cleavage and purification were performed as described above.

EXAMPLE IV

Induction of CTL By Combining CTL and T-Helper Epitopes

This example describes experiments which define the relative *in vivo* HBV-specific CTL priming efficiency of peptides expressing HBV CTL epitopes alone, CTL epitopes mixed with peptides containing T helper epitopes or CTL epitopes physically linked to T helper epitopes.

Transgenic mice (HLA-A2.1/K^b) were primed subcutaneously (base of tail) with 100 µg of peptide 875.23 (Ia^b-restricted helper epitope HBc 128-140 TPPAYRPPNAPIL) in complete Freund's adjuvant (CFA). Nine days later each of the following peptides were injected subcutaneously into two unprimed and two helper-primed mice, 100 µg/mouse in incomplete Freund's adjuvant (IFA).

EXAMPLE V

Induction of A2.1-Restricted CTL-Specific for HBenv₃₆₀₋₃₆₈

A2/K^b transgenic mice were injected with 100 microliters of an emulsion of 100 µg HBenv₃₆₀₋₃₆₈ and 100 µg HBc₁₂₈₋₁₄₀ helper epitope in incomplete Freund's adjuvant (IFA). (This emulsion was prepared by mixing 500 µg of both peptides in PBS with an equal volume of IFA.) Twenty-one days after priming, splenocytes (5x10⁶ cells/well in a 24-well plate) obtained from these animals were restimulated with syngeneic LPS blasts (2x10⁶/well) coated with the peptide HBenv₃₆₀₋₃₆₈. These effector cells (0.2x10⁶/well) were restimulated at weekly intervals. For the first and second restimulations, HBenv₃₆₀₋₃₆₈ coated LPS blasts were used, followed by HBenv₃₆₀₋₃₆₈ coated Jurkat A2.1/K^b cells. Six days after restimulation, effector cells were assayed for cytotoxicity against ⁵¹Cr labelled Jurkat A2/K^b target cells in the presence and absence of HBenv₃₆₀₋₃₆₈ (see Fig. 11).

	<u>Peptide</u>	<u>T Helper (HBc 128-140)</u>	<u>CTL (HBc 18-27)</u>
1.	875.23	TPPAYRPPNAPIL	
2.	875.15		FLPSDFFPSV
3.	875.23+875.15	TPPAYRPPNAPIL	+ FLPSDFFPSV
4.	902.01 =	TPPAYRPPNAPILFLPSDFFPSV-NH ₂	
5.	902.02	TPPAYRPPNAPILAAAFLLPSDFFPSV-NH ₂	
6.	No peptide		

Three weeks after priming with the CTL epitope, splenocytes were *in vitro* restimulated with LPS blasts coated with HBc 18-27 (coating was achieved by incubating 30 x 10⁶ LPS blasts with 100 µg of HBc 18-27 in one ml of medium; after 1-2 hr at 37°C, the cells were washed). After 6 days, effector cells were assayed for lytic activity against ⁵¹Cr labelled Jurkat A2/K^b target cells in the presence or absence of HBc 18-27.

The results showed that in 50% of the animals studied in which the T helper and CTL epitope peptides were simply mixed (i.e., not linked) and administered in an immunizing dose, induction of some detectable antigen-specific CTL activity above the level of background killing was seen. An example of the response detected is shown in Fig. 7. Surprisingly, when animals were primed with the T helper epitope linked to the CTL epitope, 100% showed evidence of specific CTL priming (Fig. 8), the magnitude of which was greater than that detected when the epitopes were administered non-linked (Fig. 7). Quite unexpectedly, as shown in Fig. 9, it was found that linking the T helper and CTL epitopes via an alanine-alanine-alanine spacer (i.e., T helper-AAA-CTL) resulted in the induction of specific CTL activity greater than that detected by linking the T helper and CTL determinants alone. Priming with the T helper peptide or CTL peptide alone did not induce HBc-specific CTL (Figs. 5 and 6). Also, prior immunization of animals to induce T helper-specific immunity did not appear to be essential for priming for CTL using either the T helper and CTL mixture or the T helper-CTL conjugate, since immunization was detected when naive animals were primed with the appropriate conjugate (Figs. 10A and B).

Example VI

Testing of linked Tetanus Toxoid T helper and HBc Cytotoxic T Cell Epitopes for In Vivo priming

Transgenic mice (HLA-A2-1/kb) were primed subcutaneously (base of the tail) with 200 mg (0.07 mM) of peptide 934.02.

<u>Peptide</u>	<u>Tetanus Toxoid 830-843 T Helper Epitope</u>	<u>Linker</u>	<u>HBV core 18-27 CTL Epitope</u>
934.02	AC-QYIKANSKFIGITE	AAA	FLPSDFFPSV

Three weeks after priming, splenocytes were restimulated *in vitro* with LPS blasts coated with HBc 18-27 (as described in example I). After 7 days cells were restimulated with jurkat A2/Kb cells coated with HBc 18-27 (as described in Example I) - After 6 days these effector cells were assayed for cytotoxicity against ⁵¹Cr labeled jurkat A2/Kb target cells in the presence or absence of HBc 18-27, Jy target cells in the presence or absence of HBc 18-27 and Jy cells transfected with HBV core. The results, shown in Fig. 12, indicate that peptide 934.02 effectively induces CTL specific for HBc 18-27. Moreover, these CTL recognize and kill endogenously presented antigen (Jy core).

Example VII

Comparison of CTL Immunity Induced by Peptide Immunization

Various modifications and formulations of an antigenic CTL peptide were tested in an effort to enhance its immunogenicity. BALB/c mice were primed subcutaneously in the base of the tail with one of the following peptides or peptide mixtures:

<u>Number</u>	<u>Peptide or Peptides</u>	<u>Dose μM/mouse</u>	<u>Formulation</u>
932.01	CTL (Flu NP 147-155) TYQRTALV	0.1,0.01	Saline, Alum, IFA
932.07	(PAM) ₂ KSS-CTL (PAM) ₂ KSSTYQRTALV	0.1,0.01	Saline, Alum, IFA
932.01 +577.01	CTL + T helper (OVA 323-336) TYQRTALV-ISQAVHAAHAEINE	0.1,0.01 ea.	Saline, Alum, IFA
932.07 +577.01	(PAM) ₂ KSS-CTL + T helper (PAM) ₂ KSSTYQRTALV-ISQAVHAAHAEINE	0.1,0.01	Saline, Alum, IFA
932.02	T helper-CTL ISQAVHAAHAEINE-TYQRTALV	0.1,0.01	Saline, Alum, IFA
932.04	(PAM) ₂ KSS-T helper-CTL (PAM) ₂ KSSISQAVHAAHAEINE-TYQRTALV	0.1,0.01	Saline, Alum, IFA
932.03	T helper-AAA-CTL ISQAVHAAHAEINE-AAA-TYQRTALV	0.1,0.01	Saline, Alum, IFA
932.05	(PAM) ₂ KSS-T helper-AAA-CTL (PAM) ₂ KSS-ISQAVHAAHAEINE-AAA-TYQRTALV	0.1,0.01	Saline, Alum, IFA

Three weeks after immunization splenocytes were removed and stimulated *in vitro* with the flu 147-155 peptide. CTL activity was assayed one week later using ⁵¹Cr-labeled B10.D2 fibroblasts as targets. Target cells were tested in the absence of antigen, in the presence of Flu 147-155 peptide or following infection with influenza PR8 virus. Representative results obtained from one of four independently run experiments are summarized in Table I.

TABLE 1. CTL immunogenicity of various modifications and formulations of PR8-NP 148-155

Peptide	Formulation					
	Saline		Alum		IFA	
	0.01 ^a	0.1	0.01	0.1	0.01	0.1
932.01	- ^b	-	-	-	+	+++
932.07	++	+	-	-	-	-
932.01 + 577.01	-	++	-	-	+++	++
932.07 + 577.01	+	-	-	-	-	±
932.02	-	-	-	+++	+++	+++
932.04	+++	+++	+++	+++	+++	+++
932.03	-	-	-	±	+++	++
932.05	+++	+++	+++	+++	+++	+++

^a = Dose (µM/mouse)

^b = CTL immunogenicity of various modifications and formulations of NP 148-155 (nucleoprotein of PR8 influenza virus). Each symbol represents the result obtained from spleen cells derived from a single Balb/c mouse and reflects the effector to target ratio (E:T) required to induce 40% antigen specific lysis of 51Cr labeled B10D2 target cells; +++ E:T below 10:1; ++ E:T between 10:1 and 30:1; + E:T greater than 30:1; - not achieved at any E:T tested.

The constructs (PAM)₂KSS-T helper-CTL and (PAM)₂KSS-T helper-AAA-CTL were superior when injected in saline or Alum compared to all of the other combinations. The peptides T helper-CTL and T helper-AAA-CTL were superior to mixing the T helper + CTL (i.e., non-linked) and worked well in IFA, but not well in saline or Alum. Thus, for vaccine development, linking the (PAM)₂KSS to a T helper peptide which is linked to the CTL peptide appears to be advantageous for inducing CTL immunity.

Example VIII

Definition of the minimal optimal sequence with the CTL peptide epitope 799.09.

Transgenic mice (A2-1/Kb) were primed with HBV virus in IFA substantially as described in Example II. Seven days after priming, splenocytes obtained from these animals were restimulated with syngeneic irradiated LPS blasts coated with peptide 799.09 (as described in Example I). After 6 cycles of restimulation with 799.09 coated cells (as described in Example II) the effector cells were cloned by limiting dilution using syngeneic irradiated LPS blasts coated with peptide 799.09 (2x10⁶ cells/well of a 96 well plate) and media supplemented with 10% rat Con A supernatant. Two CTL lines were obtained, line 110 and 113 that killed JA2Kb target cells coated with peptide 799.09. These lines were tested on a panel of 799.09 N-terminus truncated peptides and overlapping 9mers and 10mers covering the entire 799.09 sequence. As shown in Fig. 13 panel A the minimal N-terminus truncated peptide recognized by line 113 and 110 were respectively peptides HBV env. 333-348 (923.09) and 335-348 (923.07). Fig. 13 panel B shows that none of the 9mers or 10mers were recognized by line 113 implicating a longer peptide as the minimal sequence required for recognition by this

CTL line. The minimal sequence recognized by line 110 is represented by peptides HBV env. 333-341 (923.26) and HBV env 335-343 (923.22) indicating that possibly two distinct but overlapping peptides can serve as antigenic determinants for 799.09 specific CTL.

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

Claims

10

1. A CTL inducing peptide comprising from six to nineteen amino acids wherein at least a majority of the amino acids of said CTL inducing peptide are homologous to a corresponding portion of 799.10 (HBenv₃₄₉₋₃₆₈) having the following sequence:

15

799.10 (HBenv₃₄₉₋₃₆₈) [Seq. ID No. 2]
Leu-Ser-Pro-Thr-Val-Trp-Leu-Ser-Val-Ile-
Trp-Met-Met-Trp-Tyr-Trp-Gly-Pro-Ser-Leu.

20

2. The peptide of claim 1, which is

25

884.02 (HBenv₃₄₉₋₃₅₈) [Seq. ID No. 3]
Leu-Ser-Pro-Thr-Val-Trp-Leu-Ser-Val-Ile.

3. The peptide of claim 1, which is

30

917.07 (HBenv₃₆₀₋₃₆₈)
Trp-Met-Met-Trp-Tyr-Trp-Gly-Pro-Ser-Leu.

35

4. A CTL inducing peptide comprising from six to twenty amino acids wherein at least a majority of the amino acids of said CTL inducing peptide are homologous to a corresponding portion of 799.09 (HBenv₃₂₃₋₃₄₈) having the following sequence:

40

799.09 (HBenv₃₂₉₋₃₄₈) [Seq. ID No. 2]
Ala-Ser-Ala-Arg-Phe-Ser-Trp-Leu-Ser-Leu-Leu-
Val-Pro-Phe-Val-Gln-Trp-Phe-Val-Gly.

5. The peptide of claim 4, which is

45

923.22 (HBenv₃₃₅₋₃₄₃) [Seq. ID No. 3]
Trp-Leu-Ser-Leu-Leu-Val-Pro-Phe-Val.

6. The peptide of claim 4, which is

50

923.27 (HBenv₃₃₂₋₃₄₁) [Seq. ID No. 3]
Arg-Phe-Ser-Trp-Leu-Ser-Leu-Leu-Val-Pro.

55

7. A CTL/T helper peptide conjugate comprising a CTL inducing peptide linked to a T helper peptide.
8. The CTL/T helper peptide conjugate of claim 7 wherein the CTL inducing peptide is 799.09 (HBenv₃₂₃₋₃₄₈), 799.10 (HBenv₃₄₉₋₃₆₈), 799.08 (HBenv₃₀₉₋₃₂₈), 923.27 (HBenv₃₃₂₋₃₄₁), 917.07

(HBenv₃₆₀₋₃₆₈), or 875.15 (HBc₁₈₋₂₇).

9. The CTL/T helper peptide conjugate of claim 7, wherein the T helper peptide is hepatitis B core peptide HBc₁₋₂₀, HBc₅₀₋₆₉, HBc₁₁₁₋₁₂₅, tetanus toxoid (TT₈₃₀₋₈₄₃), tetanus toxoid (TT₈₃₀₋₈₄₃).

10. A CTL/T helper peptide conjugate wherein the CTL inducing peptide is 875.15 (HBc₁₈₋₂₇).

11. The CTL/T helper peptide conjugate of claim 10, wherein the CTL inducing peptide is linked to the T helper peptide by a spacer molecule.

12. The CTL/T helper peptide conjugate of claim 11, wherein the spacer molecule is comprised of from one to ten amino acids.

13. The CTL/T helper peptide conjugate of claim 12, wherein the spacer is Ala-Ala-Ala.

14. A CTL inducing peptide comprising from six to twenty amino acids wherein at least a majority of the amino acids of said CTL inducing peptide are homologous to a corresponding portion of 799.08 (HBenv₃₀₉₋₃₂₈) having the following sequence:

799.08 (HBenv₃₀₉₋₃₂₈) [Seq. ID No. 1]
Asn-Cys-Thr-Cys-Ile-Pro-Ile-Pro-Ser-Ser-Trp-Ala-
Phe-Gly-Lys-Phe-Leu-Trp-Glu-Trp.

15. A CTL inducing peptide comprising from six to twenty amino acids wherein at least a majority of the amino acids of said CTL inducing peptide are homologous to the corresponding portion of 802.03 (HBc₉₁₋₁₁₀) having the following sequence:

802.03 (HBc₉₁₋₁₁₀) [Seq. ID No. 4]
Thr-Asn-Met-Gly-Leu-Lys-Phe-Arg-Gln-Leu-
Leu-Trp-Phe-His-Ile-Ser-Cys-Leu-Thr-Phe.

16. The peptide of claim 15, which is HBc₉₂₋₁₀₁ (Asn-Met-Gly-Leu-Lys-Phe-Arg-Gln-Leu-Leu), HBc₉₂₋₁₀₀ (Asn-Met-Gly-Leu-Lys-Phe-Arg-Gln-Leu), or HBc₉₃₋₁₀₂ (Met-Gly-Leu-Lys-Phe-Arg-Gln-Leu-Leu-Trp).

17. A pharmaceutical composition for the treatment of hepatitis B virus infection, which comprises a CTL inducing peptide according to any one of claims 1-16 and a pharmaceutically acceptable carrier.

18. The pharmaceutical composition of claim 17, wherein the CTL inducing peptide is 875.15 (HBc₁₈₋₂₇).

19. The pharmaceutical composition of claim 17, wherein the carrier is a liposome.

20. The pharmaceutical composition of claim 17, wherein the peptide is conjugated to the carrier.

21. The pharmaceutical composition of claim 20, wherein the carrier is a lipid.

22. The pharmaceutical composition of claim 21, wherein the lipid is comprised of palmitic acid attached to epsilon and alpha amino groups of a Lys residue, wherein the Lys is linked to the amino terminus of the peptide by means of a Ser-Ser linker.

23. A method for identifying an individual susceptible to developing chronic hepatitis B virus infection, comprising:

incubating lymphomononuclear cells from an individual of interest with a hepatitis B peptide antigen according to any one of claims 1-6 or 14-16 which induces a HLA class I-restricted CTL response to hepatitis B virus; and

determining therefrom the ability of the individual to mount the CTL response to the antigen and thus susceptibility to developing chronic hepatitis B virus infection.

24. The method of claim 23, wherein the individual of interest suffers from acute hepatitis B infection.
25. A peptide according to claims 1-6 or 14-16 which is expressed by a DNA construct that comprises a transcriptional promotor, a DNA sequence encoding said peptide, and a transcription terminator, each operably linked for expression of said peptide.
26. A CTL/T helper/lipid conjugate comprising a linked CTL inducing peptide, T helper peptide and a lipid.
27. The CTL/T helper/lipid conjugate of claim 26, wherein the lipid is linked to the N terminus of the T helper peptide which is linked at its carboxy terminal end to the CTL inducing peptide.
28. The CTL/T helper/lipid conjugate of claim 27, wherein the lipid is linked to the N terminus of the T helper peptide by a spacer molecule.
29. The CTL/T helper/lipid conjugate of claim 27, wherein the spacer molecule is Lys-Ser-Ser.
30. The CTL/T helper/lipid conjugate of claim 27, wherein the CTL inducing peptide is linked to the T helper peptide by a spacer molecule.
31. The CTL/T helper/lipid conjugate of claim 30, wherein the spacer molecule between the CTL inducing peptide and the T helper peptide is Ala-Ala-Ala.
32. The CTL/T helper/lipid conjugate of claim 56, wherein CTL inducing peptide is linked to the T helper peptide by a spacer molecule.
33. The CTL/T helper/lipid conjugate of claim 32, wherein the spacer molecule between the CTL inducing peptide and the T helper peptide is Ala-Ala-Ala.
34. The CTL/T helper/lipid conjugate of claim 36, wherein CTL inducing peptide is HBc18-27.

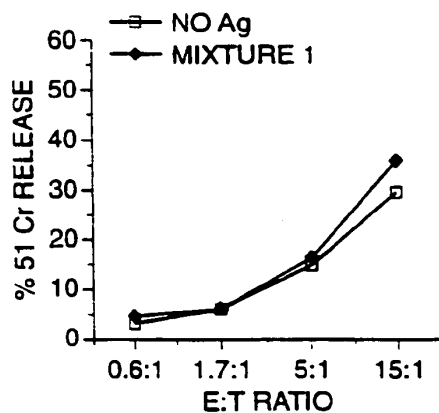


FIG. 1a

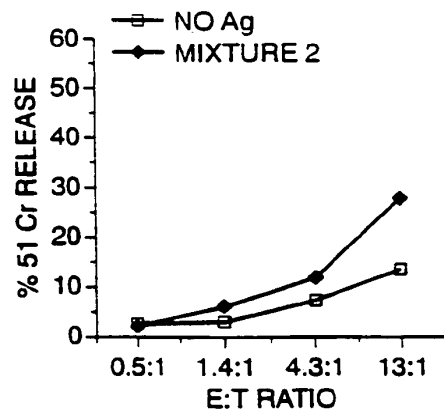


FIG. 1b

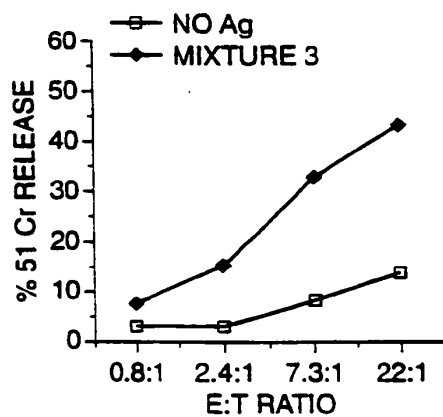


FIG. 1c

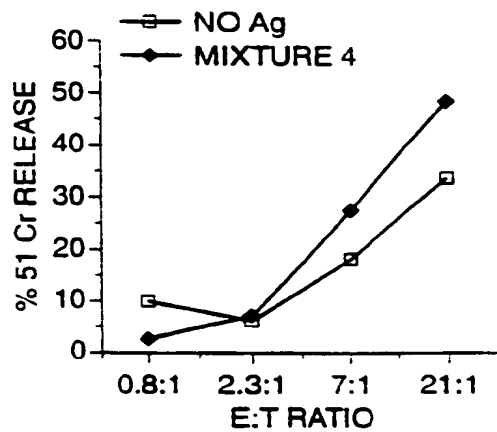


FIG. 1d

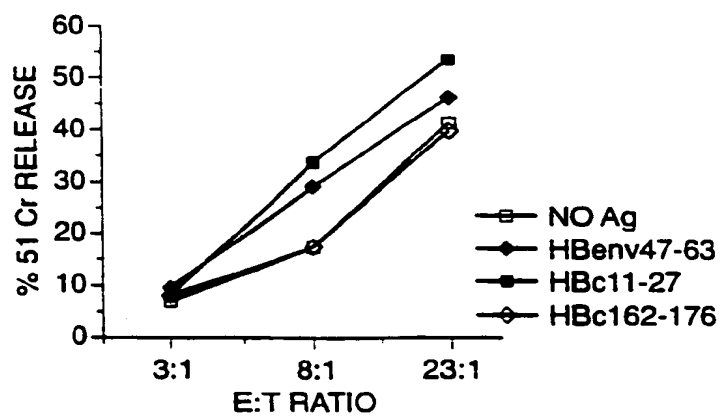


FIG. 1e

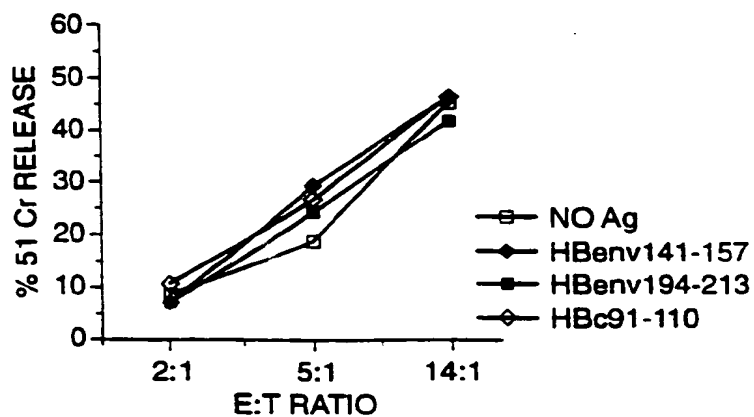


FIG. 1f

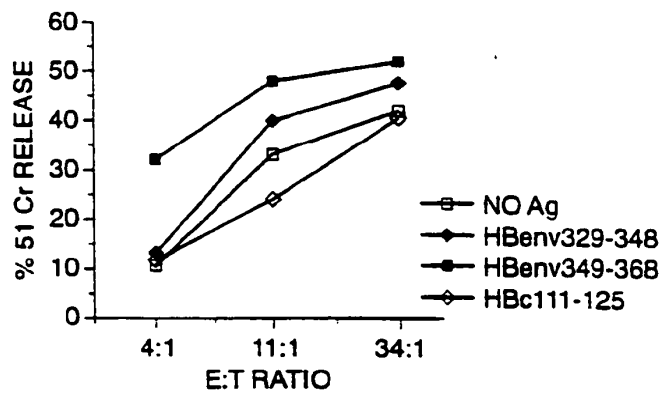


FIG. 1g

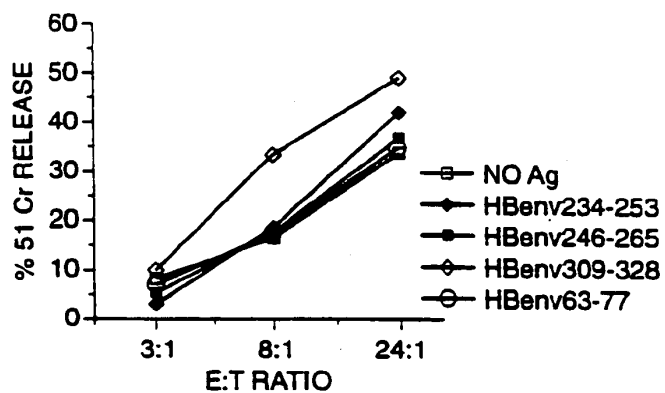


FIG. 1h

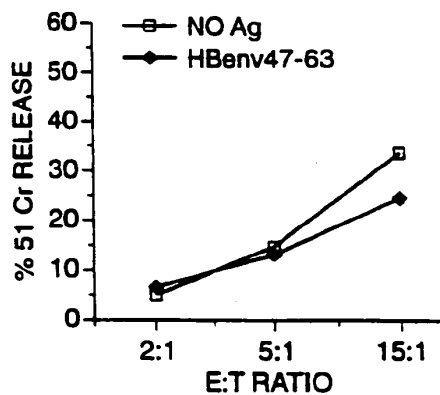


FIG. 2i

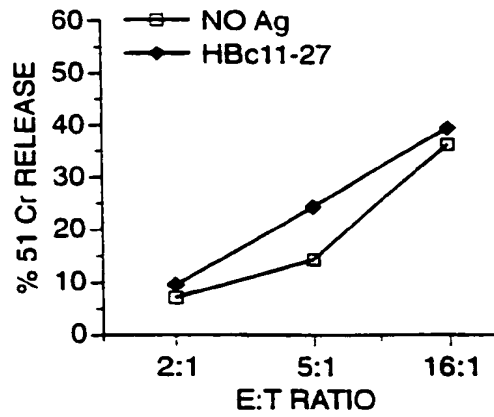


FIG. 2j

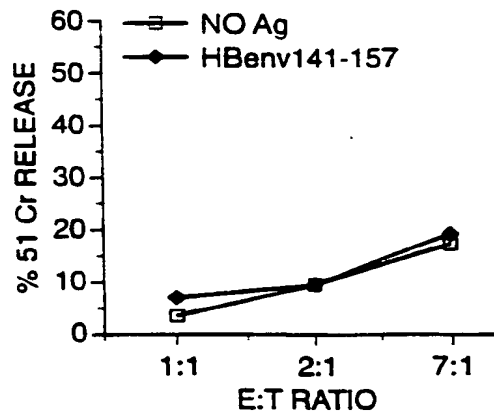


FIG. 2k

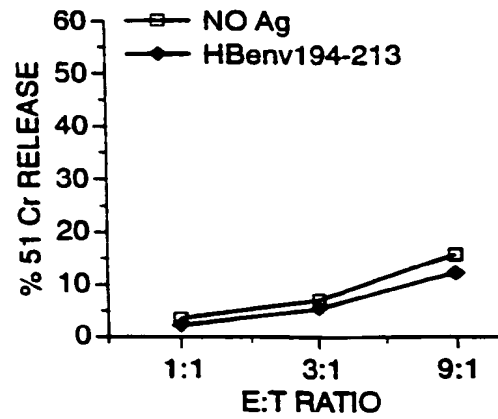


FIG. 2l

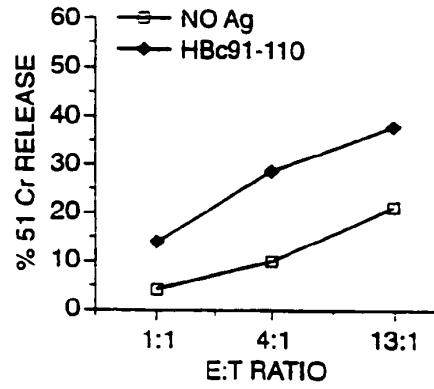


FIG. 2m

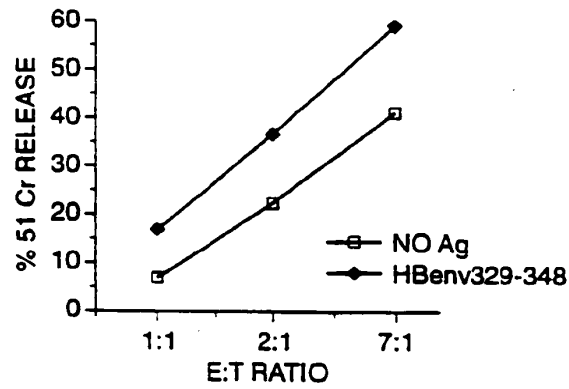


FIG. 2n

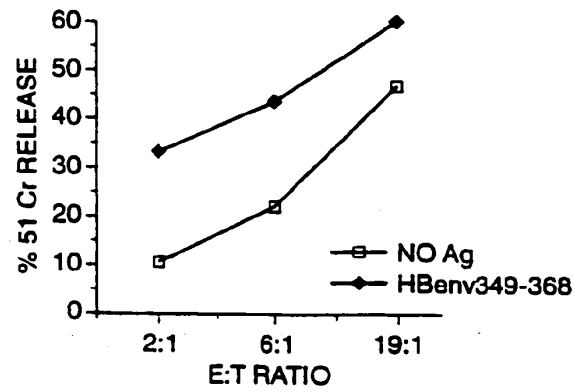


FIG. 2o

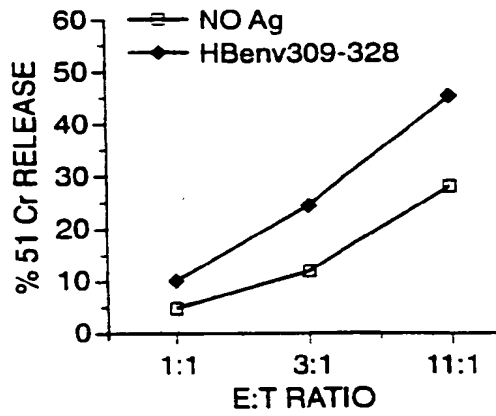


FIG. 2p

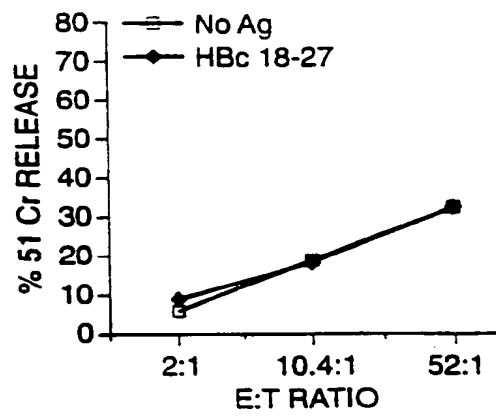


FIG. 3a

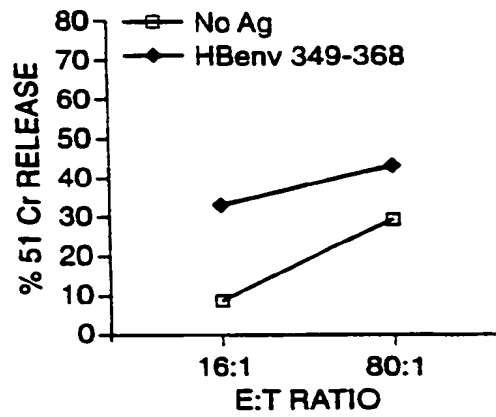


FIG. 3b

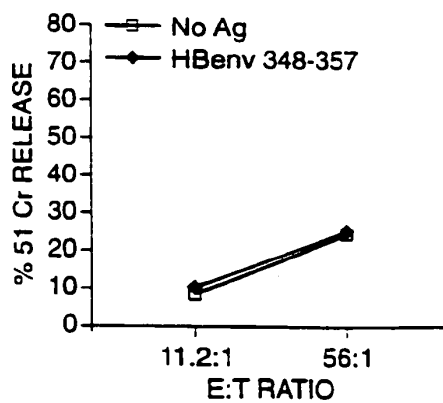


FIG. 3c

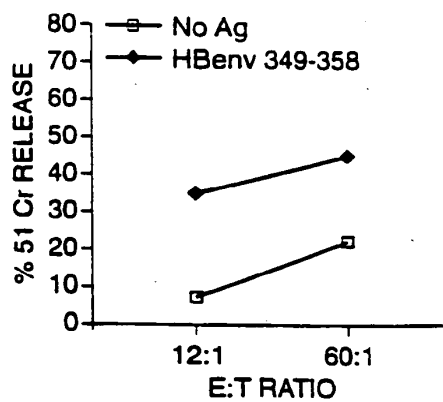


FIG. 3d

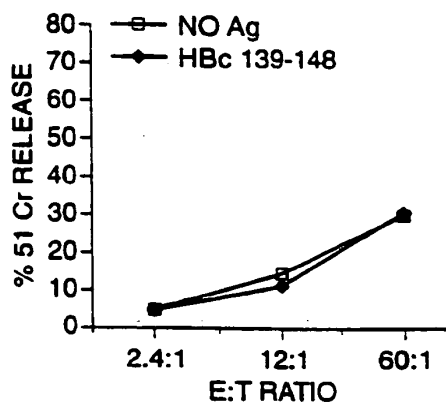


FIG. 3e

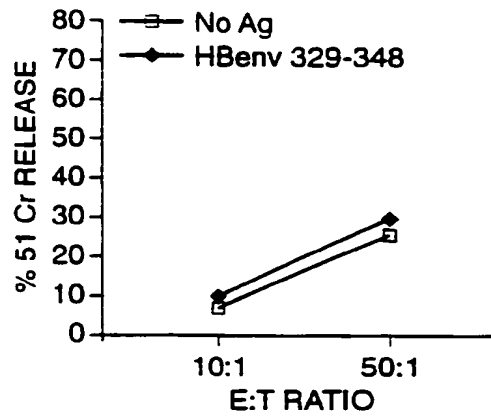


FIG. 3f

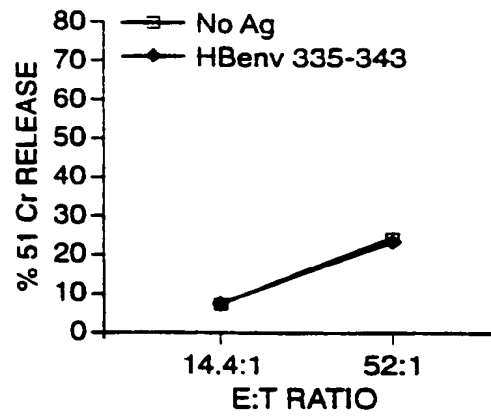


FIG. 3g

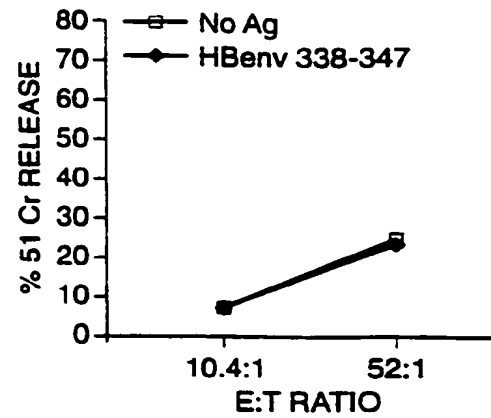


FIG. 3h

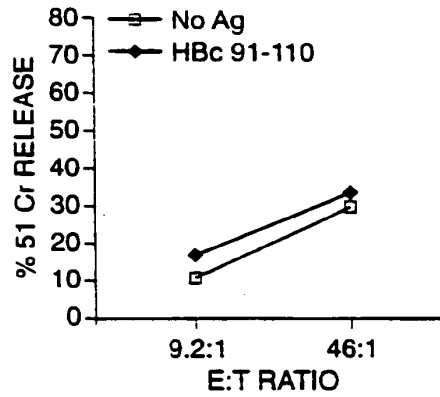


FIG. 3i

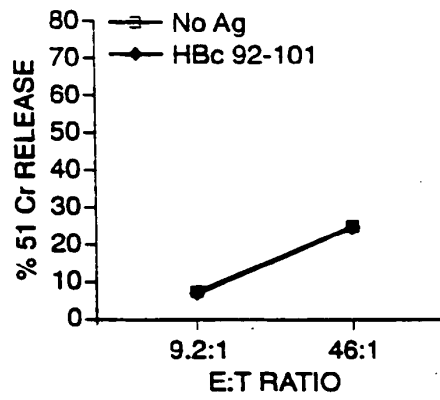


FIG. 3j

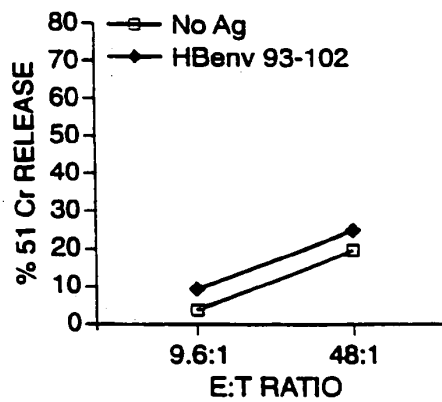


FIG. 3k

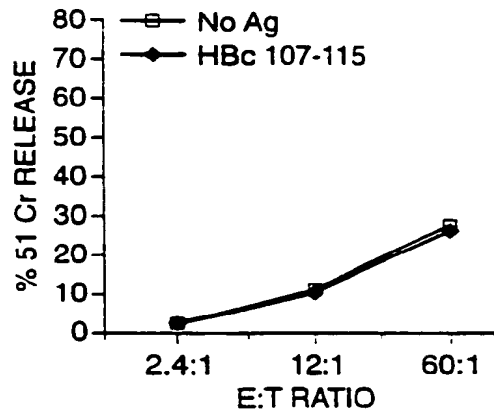


FIG. 3I

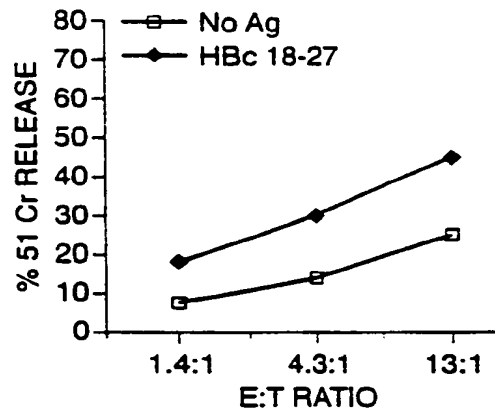


FIG. 4a

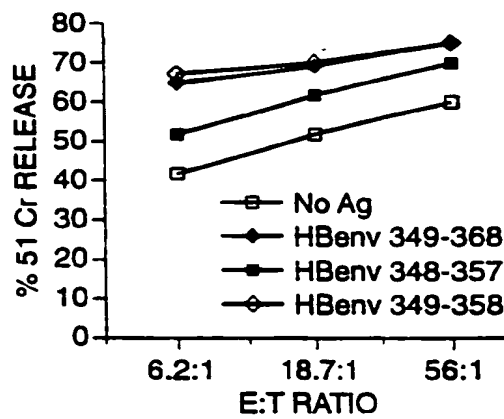


FIG. 4b

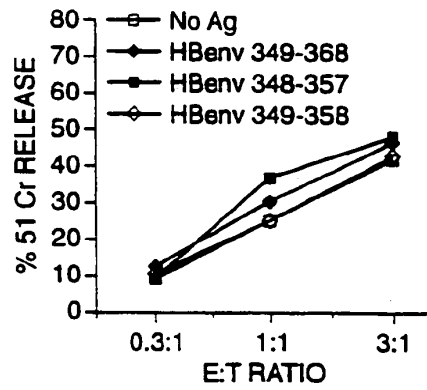


FIG. 4c

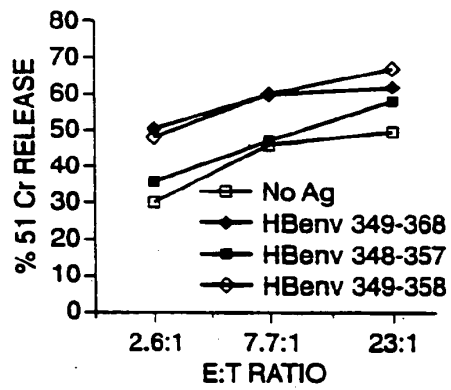


FIG. 4d

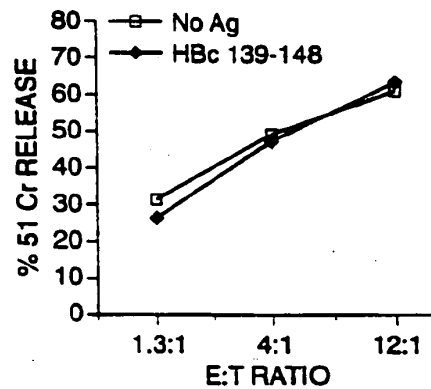


FIG. 4e

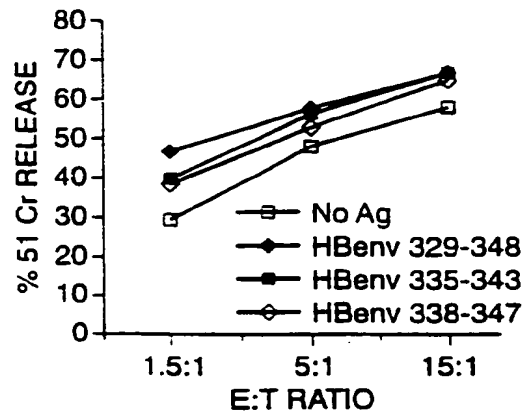


FIG. 4f

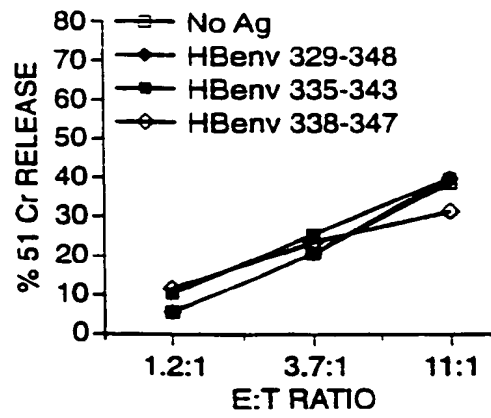


FIG. 4g

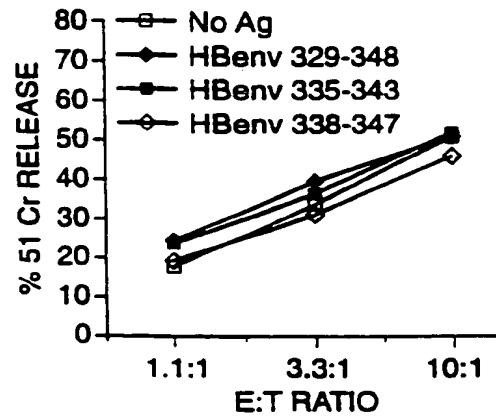


FIG. 4h

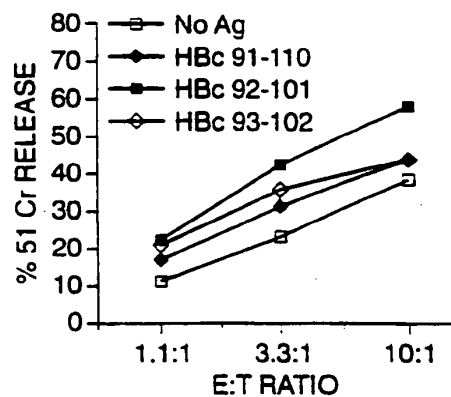


FIG. 4i

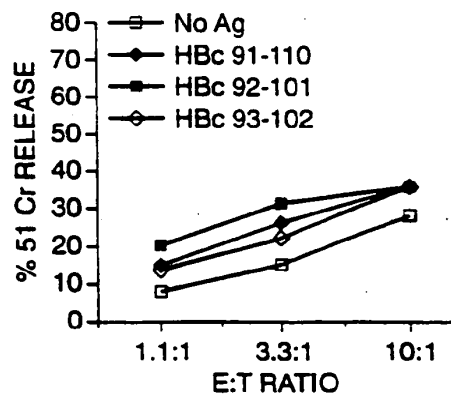


FIG. 4j

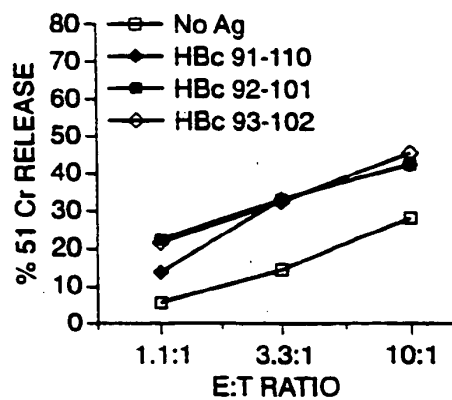


FIG. 4k

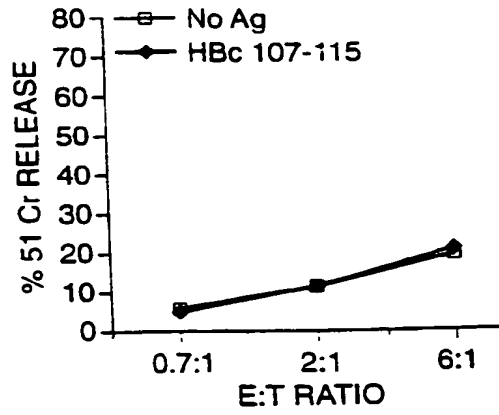


FIG. 4I

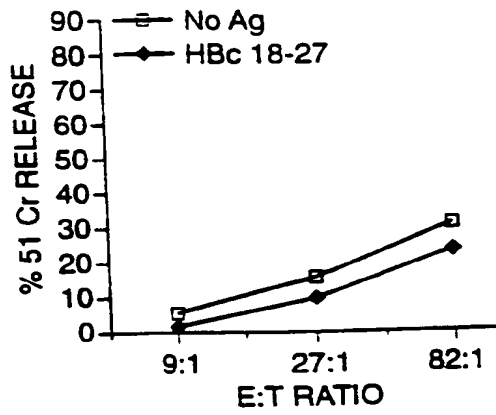


FIG. 5

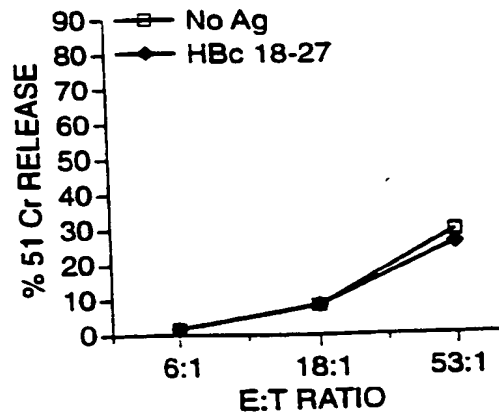


FIG. 6

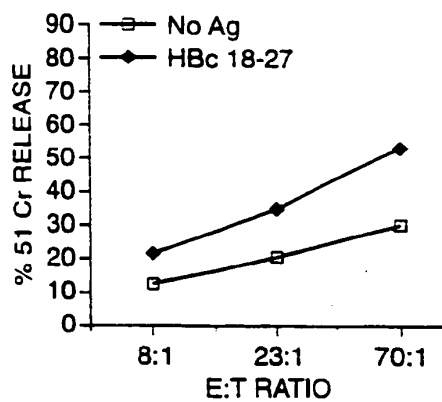


FIG. 7

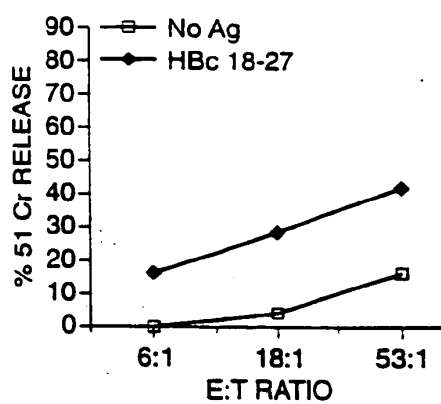


FIG. 8

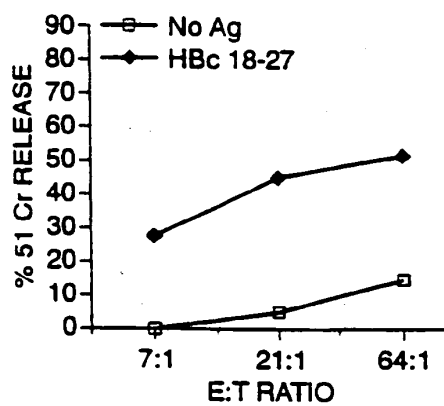


FIG. 9

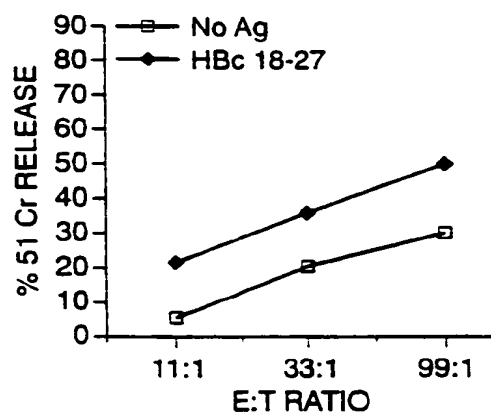


FIG. 10a

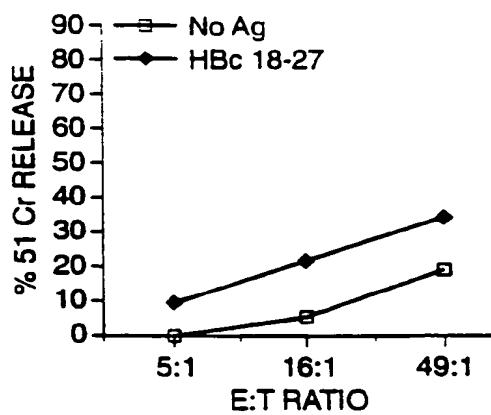


FIG. 10b

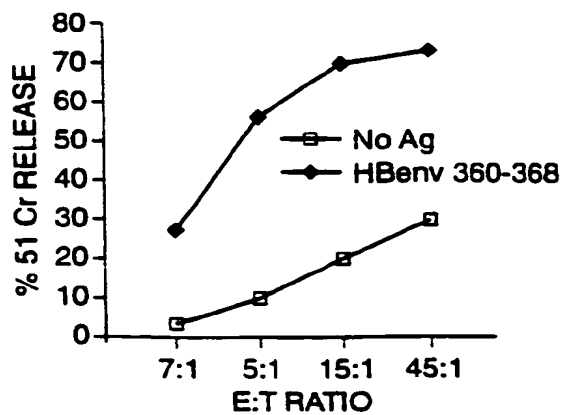


FIG. 11

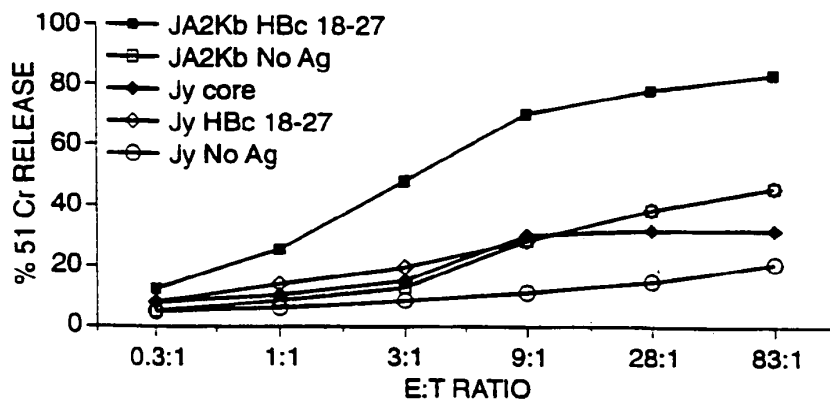


FIG. 12

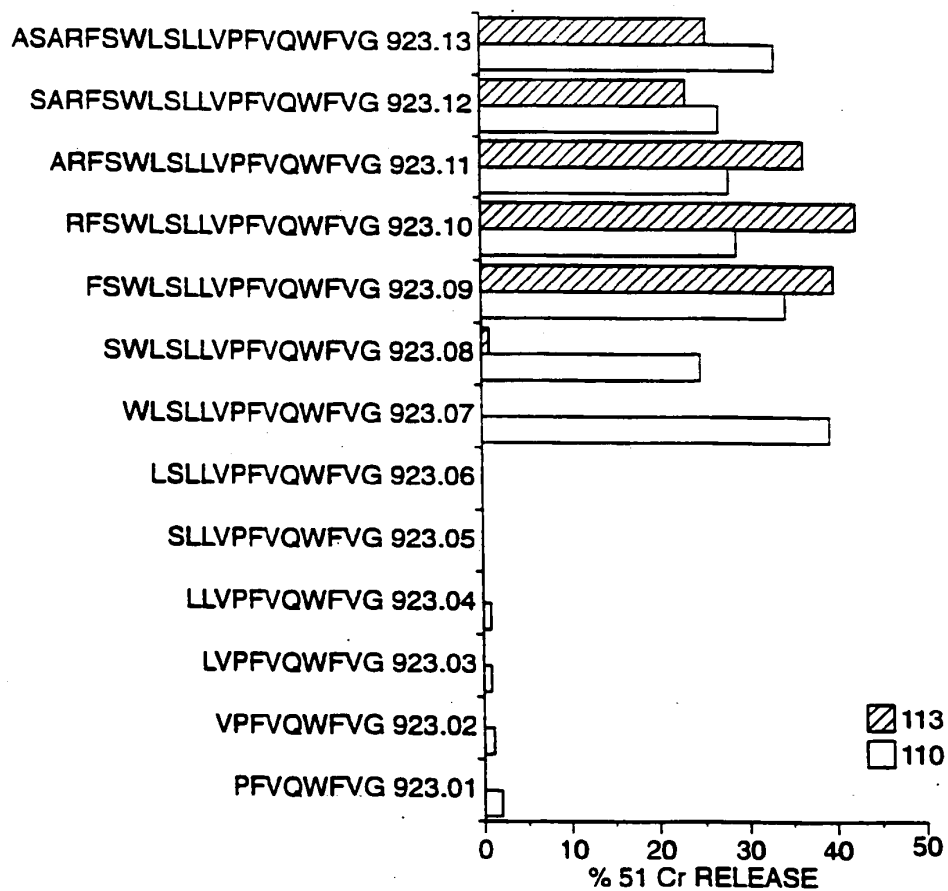


FIG. 13a

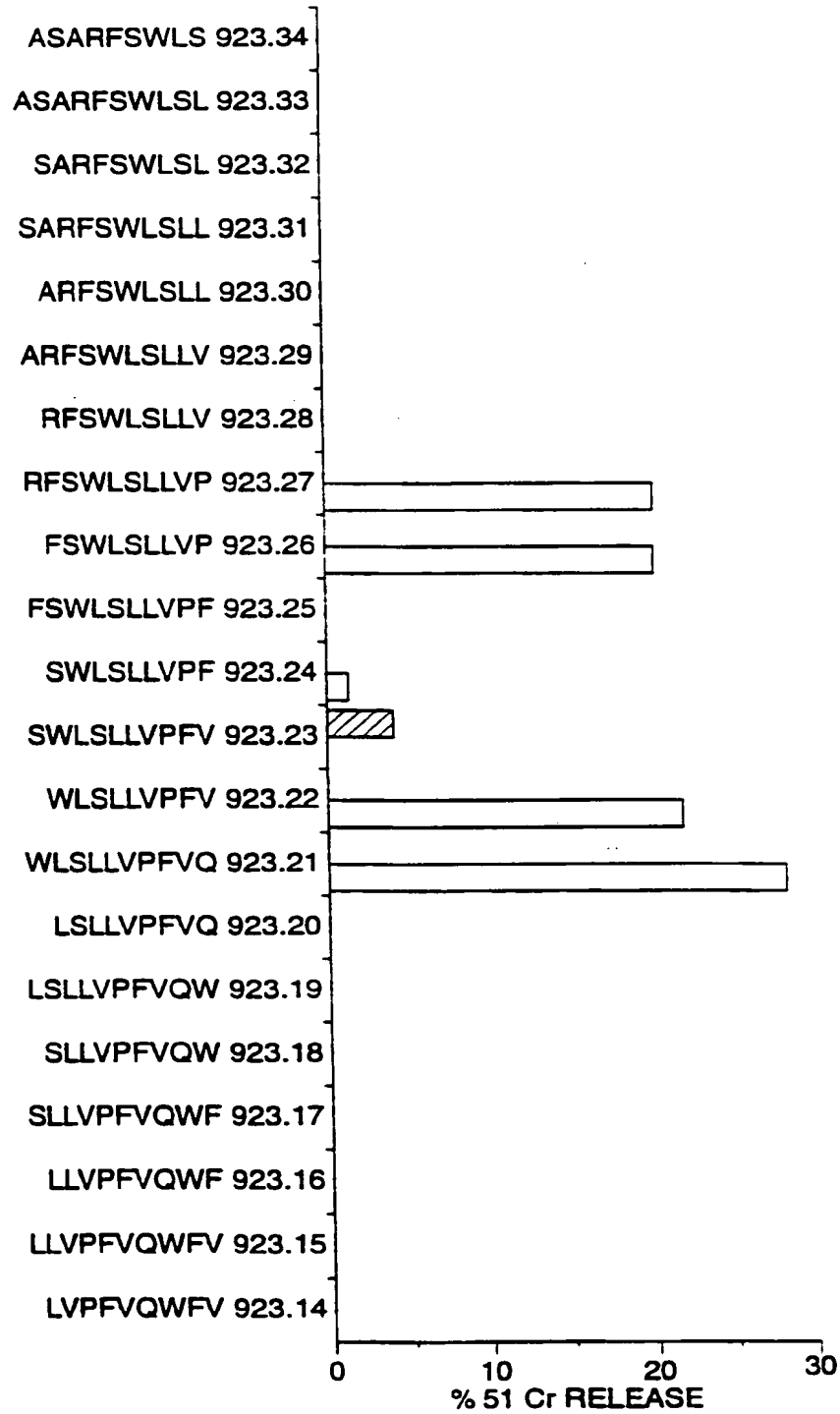


FIG. 13b

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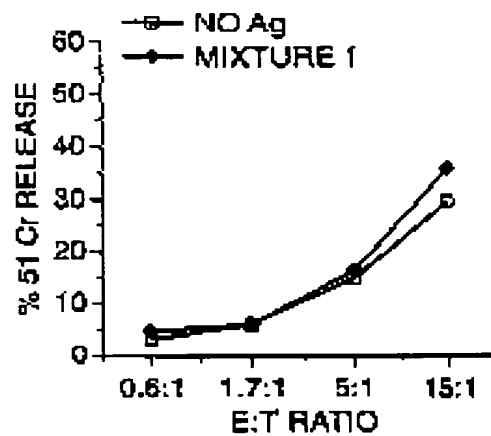


FIG. 1a

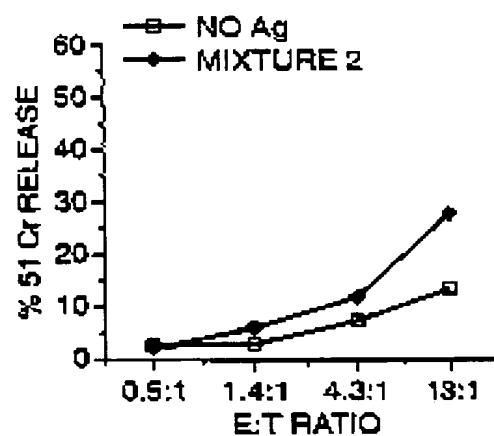


FIG. 1b

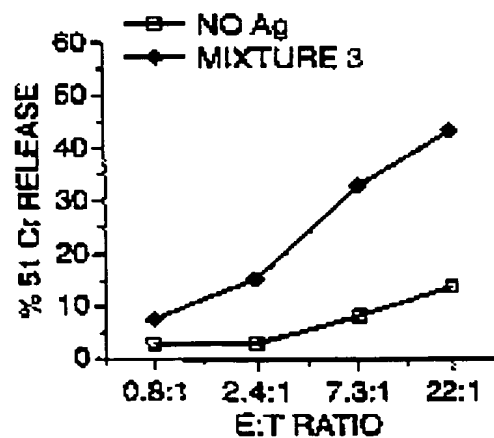


FIG. 1c

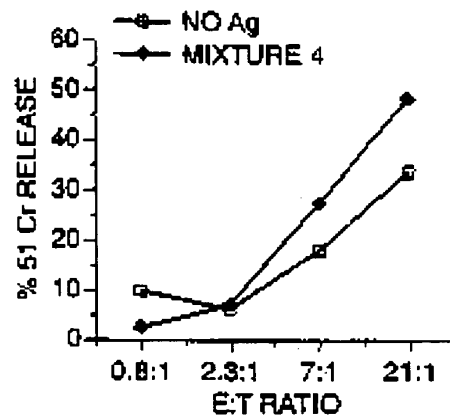


FIG. 1d

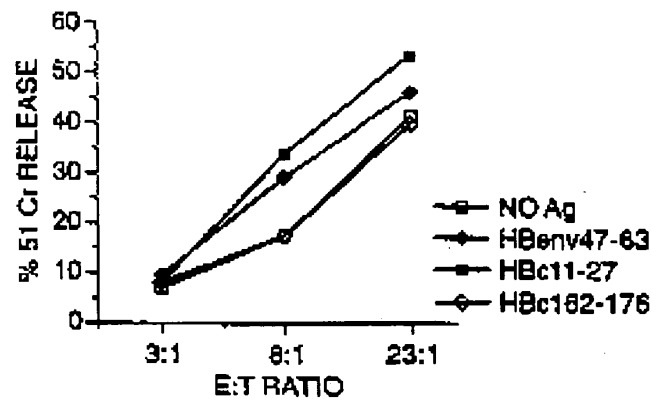


FIG. 1e

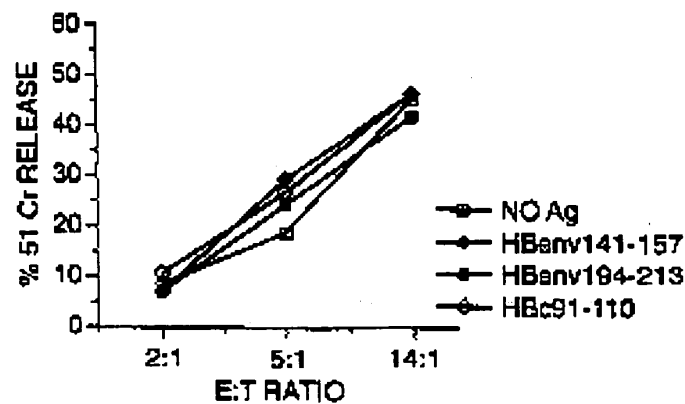


FIG. 1f

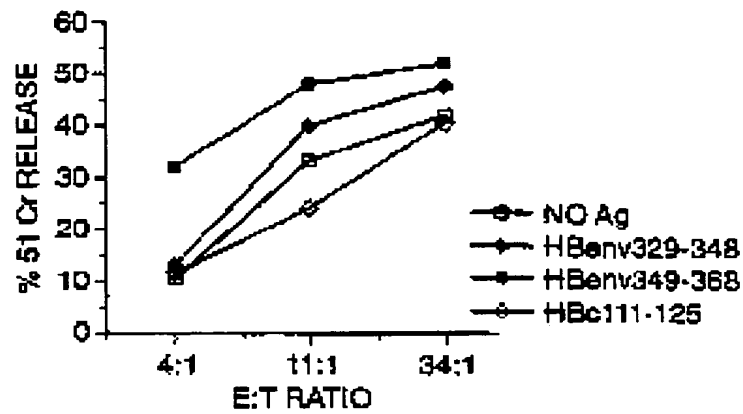


FIG. 1g

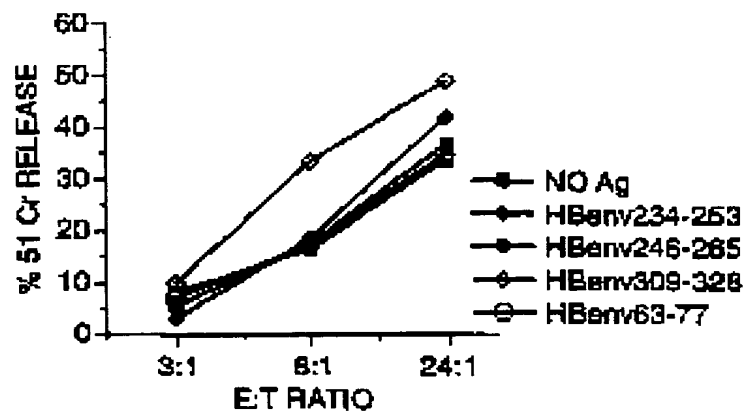


FIG. 1h

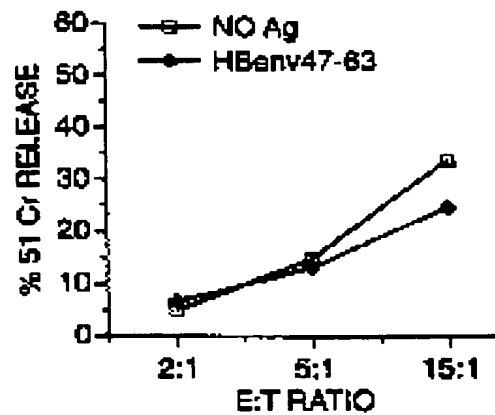


FIG. 2i

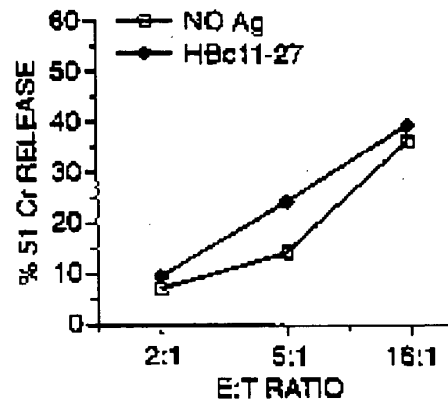


FIG. 2j

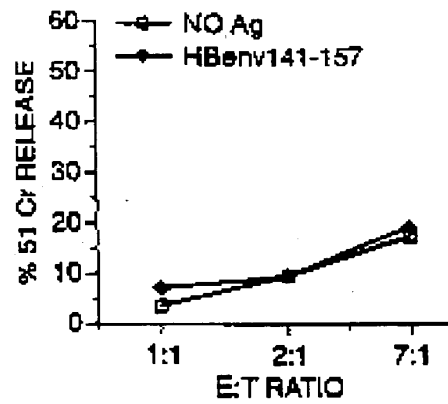


FIG. 2k

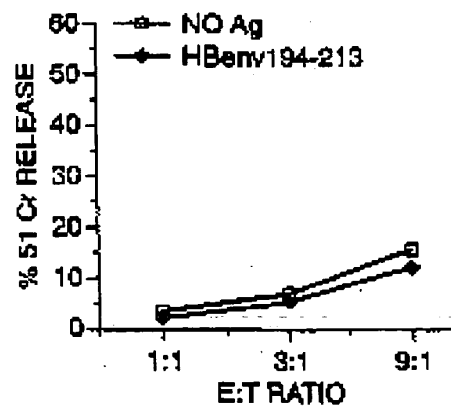


FIG. 2l

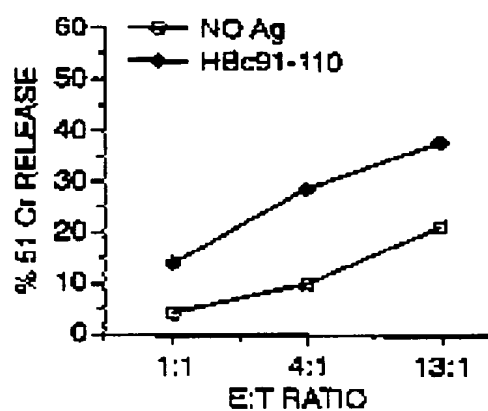


FIG. 2m

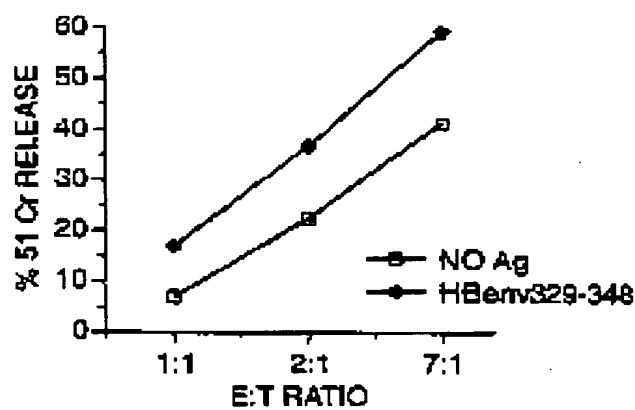


FIG. 2n

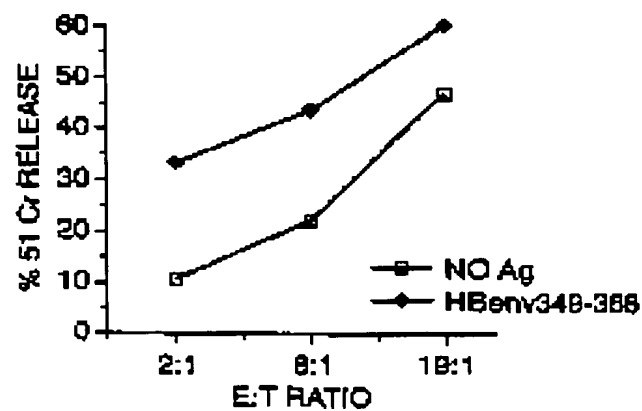


FIG. 2o

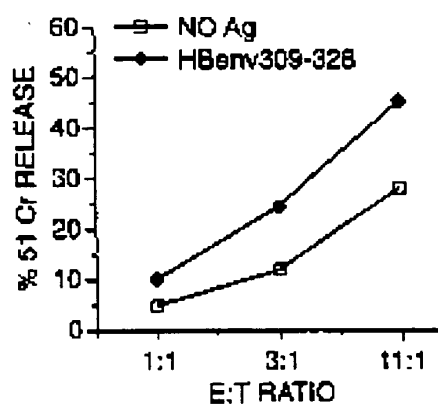


FIG. 2p

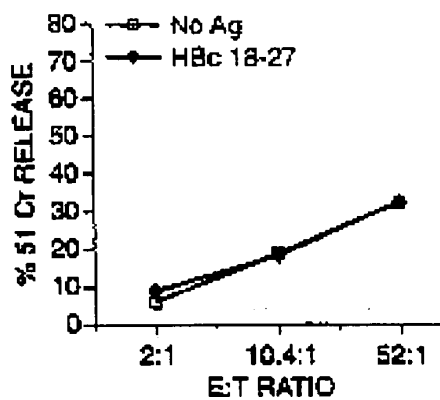


FIG. 3a

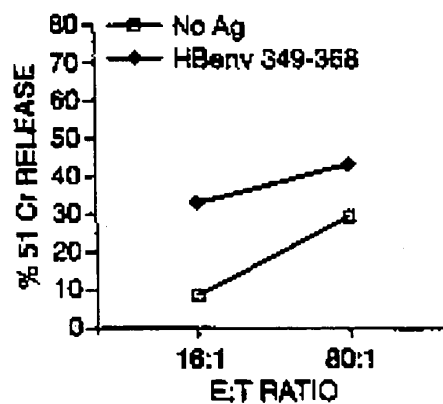


FIG. 3b

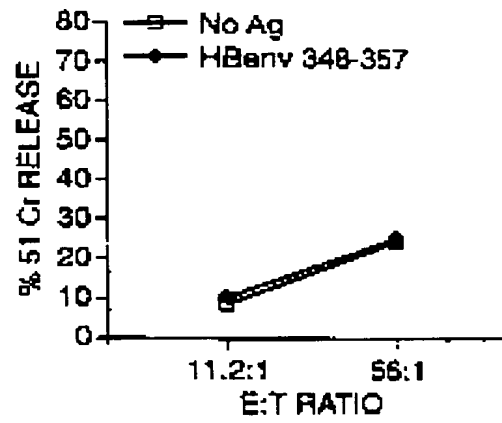


FIG. 3c

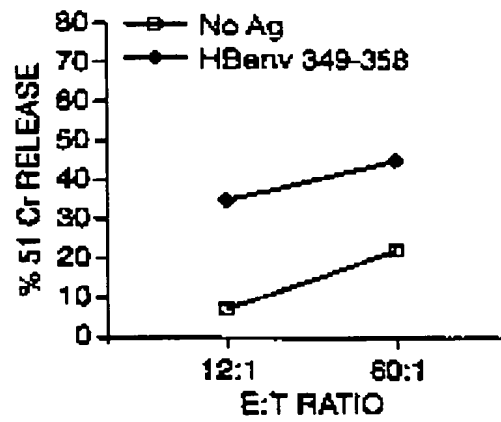


FIG. 3d

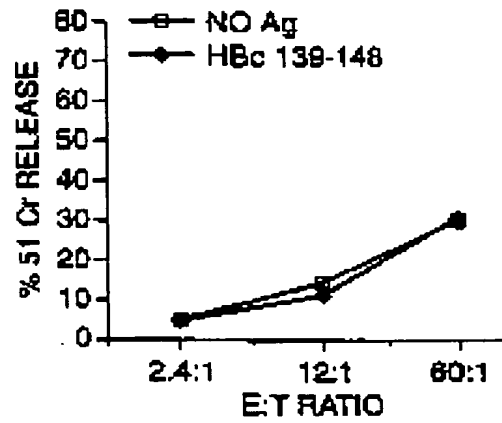


FIG. 3e

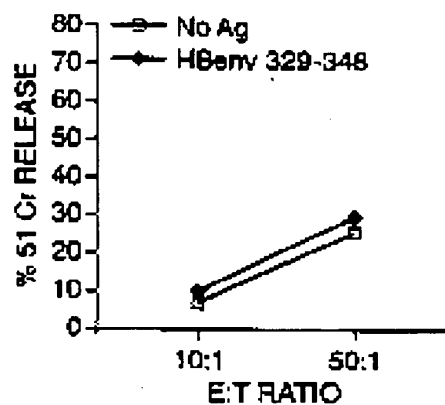


FIG. 3f

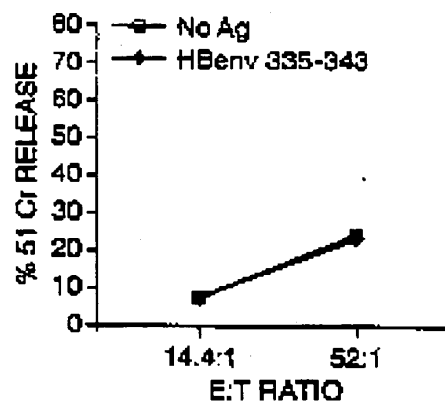


FIG. 3g

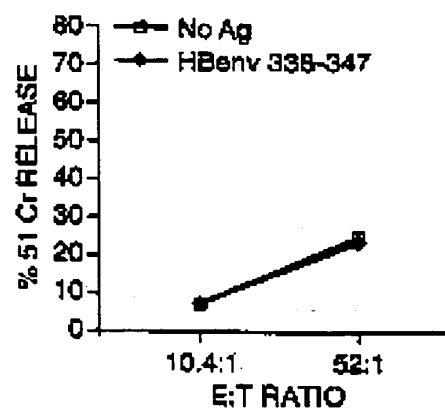


FIG. 3h

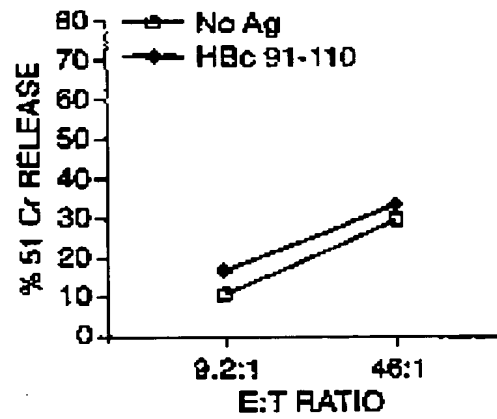


FIG. 3i

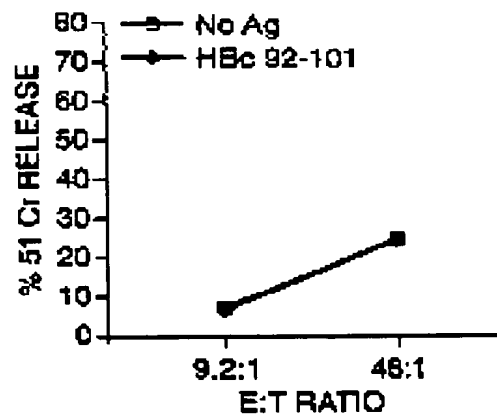


FIG. 3j

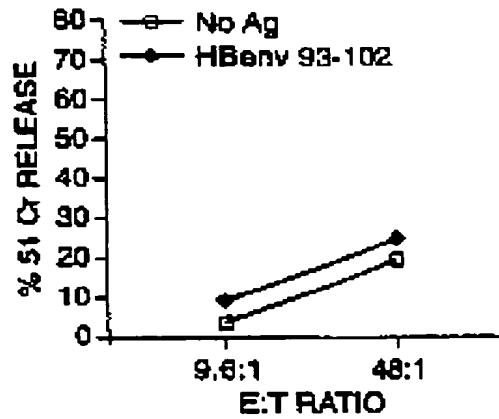


FIG. 3k

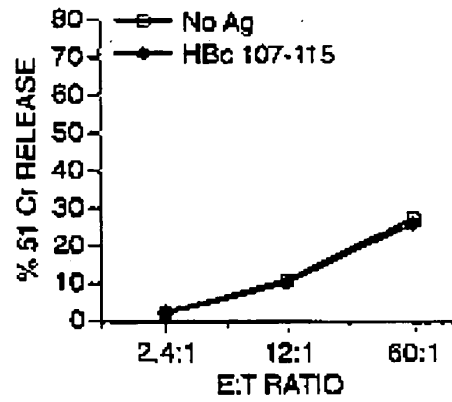


FIG. 3f

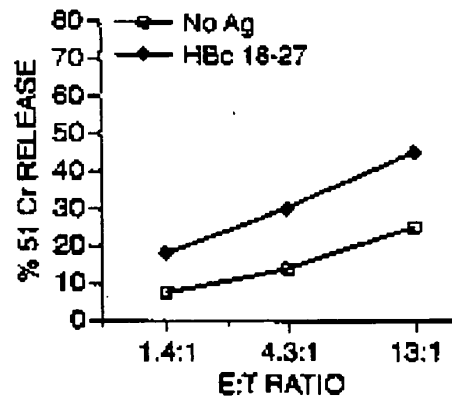


FIG. 4a

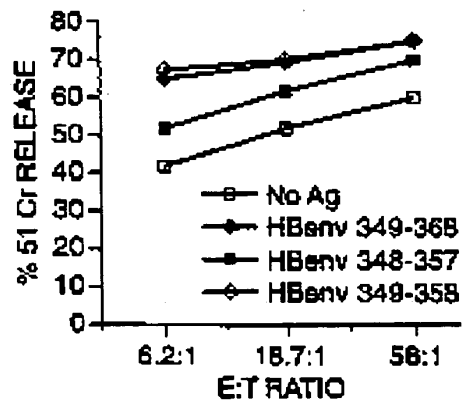


FIG. 4b

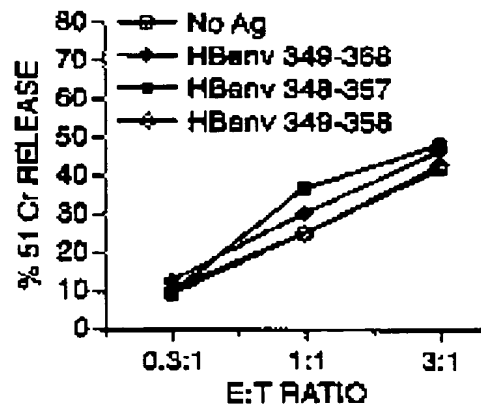


FIG. 4c

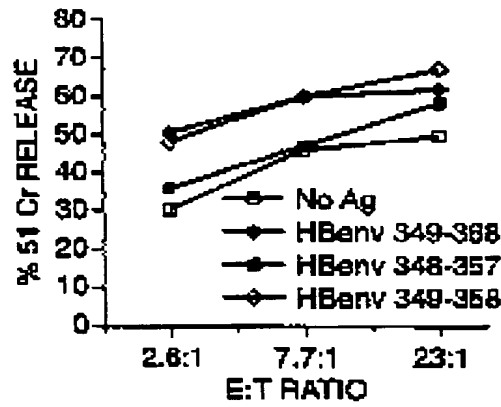


FIG. 4d

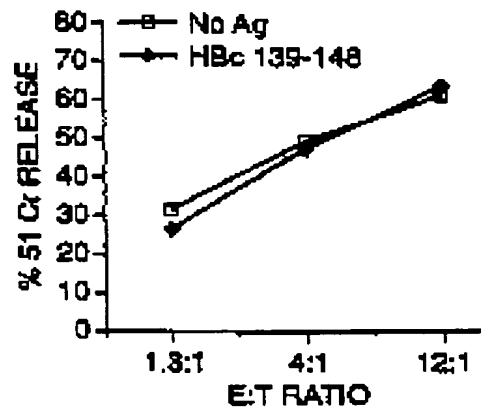


FIG. 4e

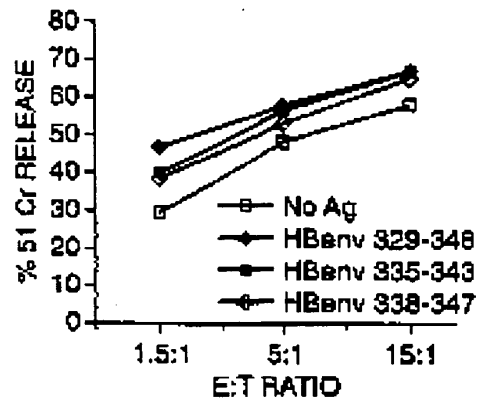


FIG. 4f

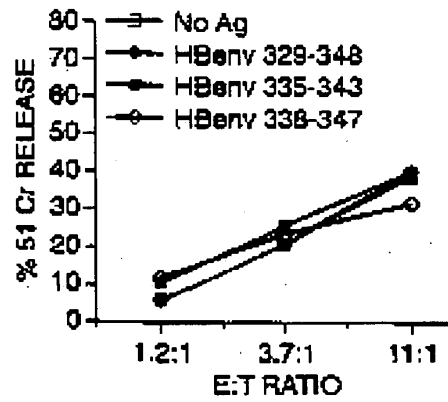


FIG. 4g

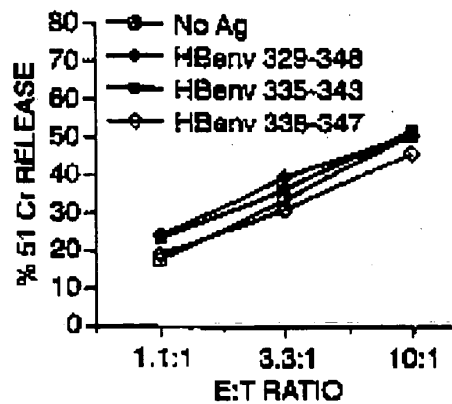


FIG. 4h

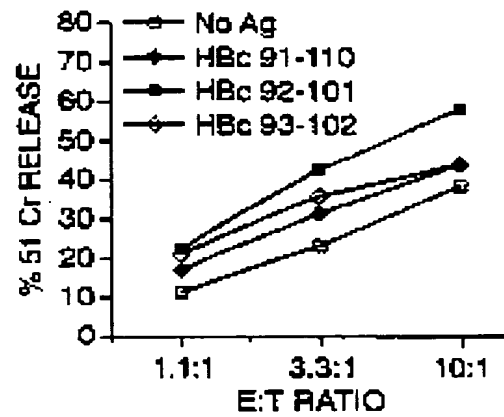


FIG. 4i

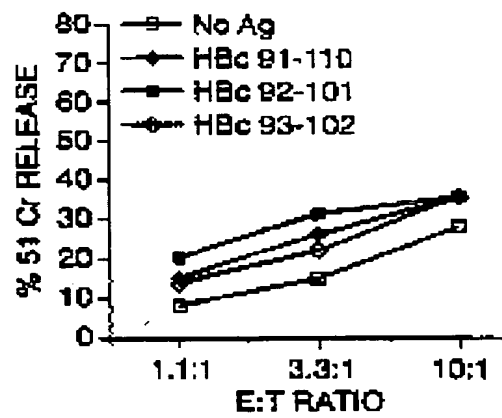


FIG. 4j

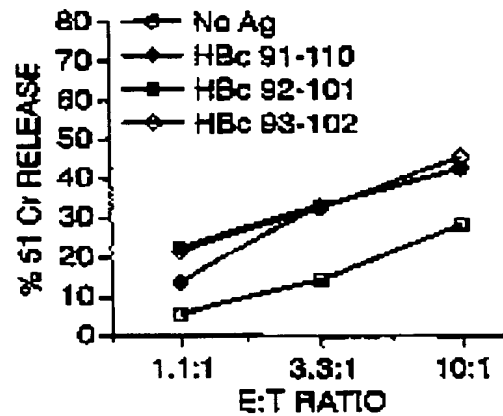


FIG. 4k

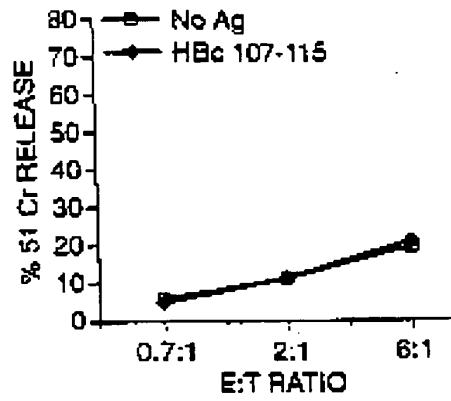


FIG. 4I

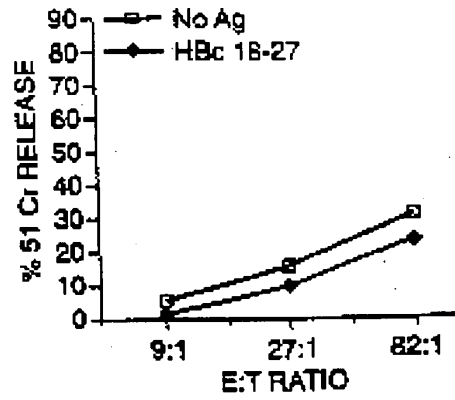


FIG. 5

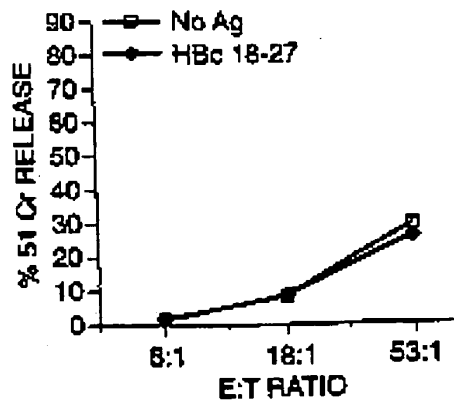


FIG. 6

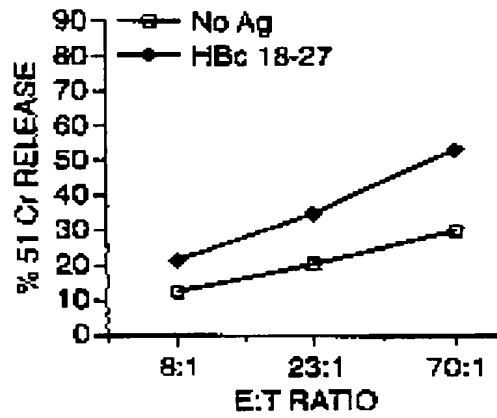


FIG. 7

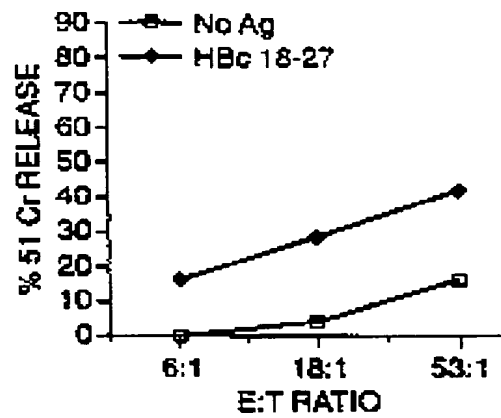


FIG. 8

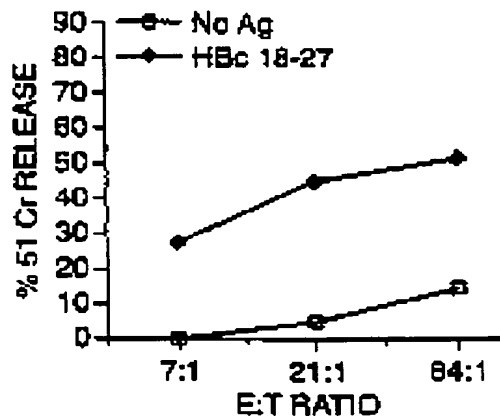


FIG. 9

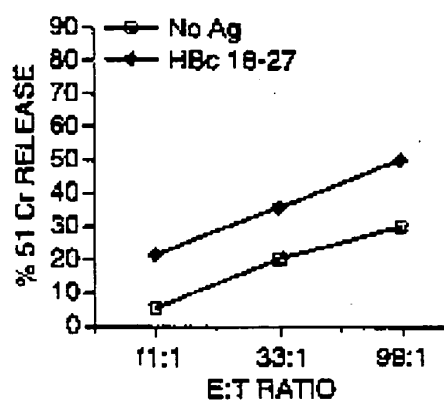


FIG. 10a

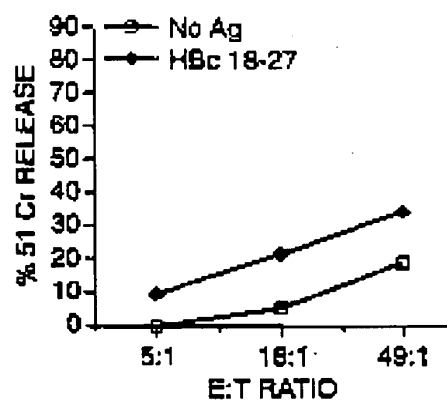


FIG. 10b

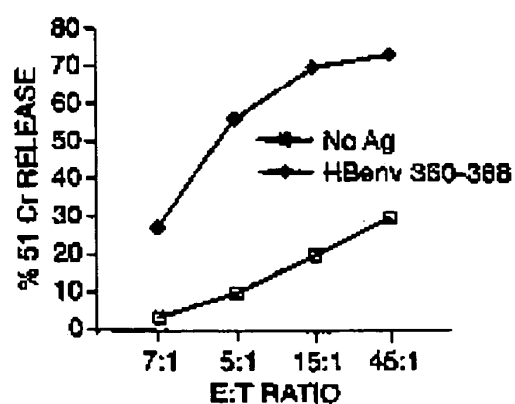


FIG. 11

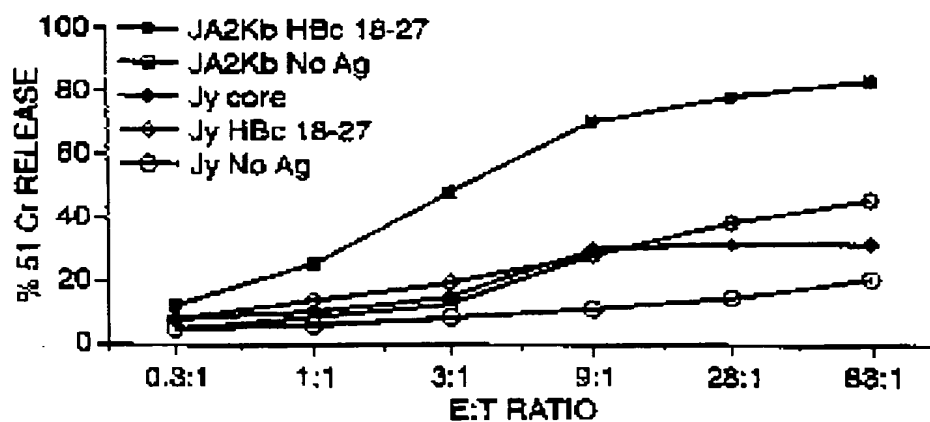


FIG. 12

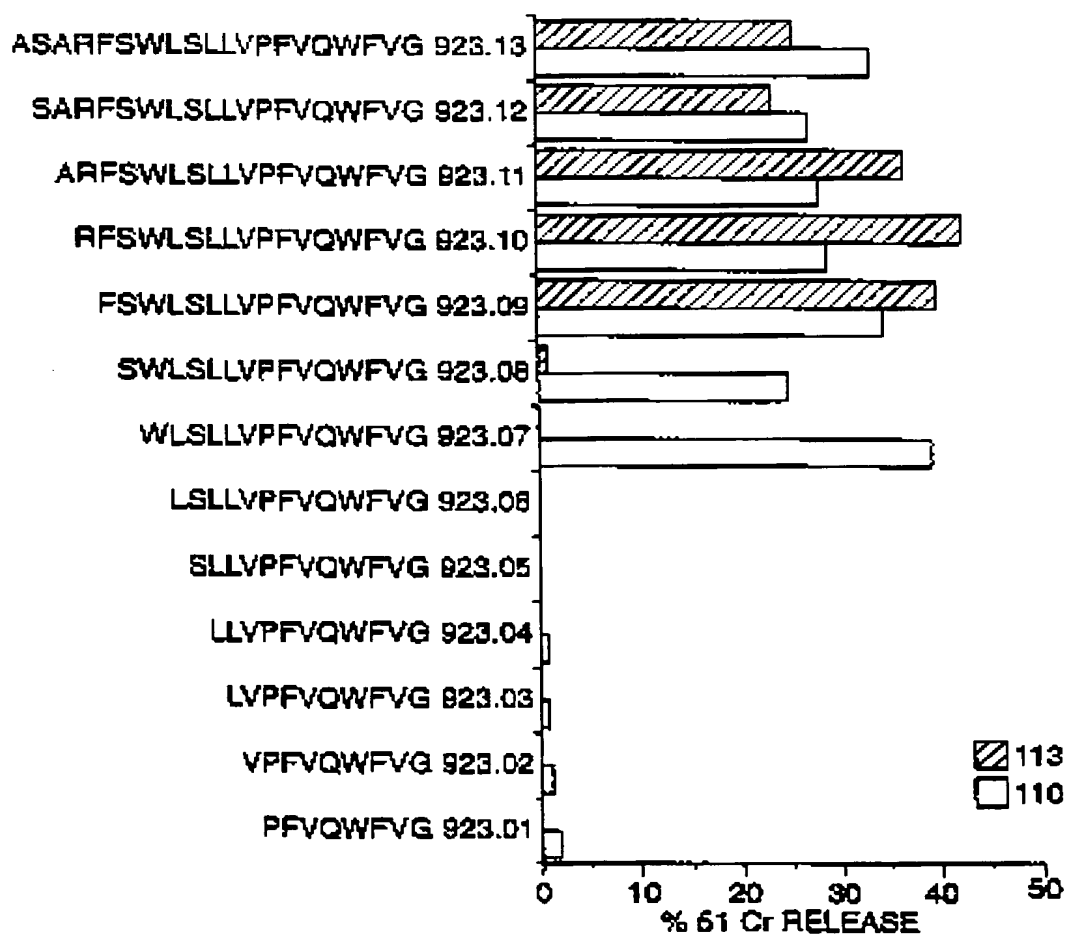


FIG. 13a

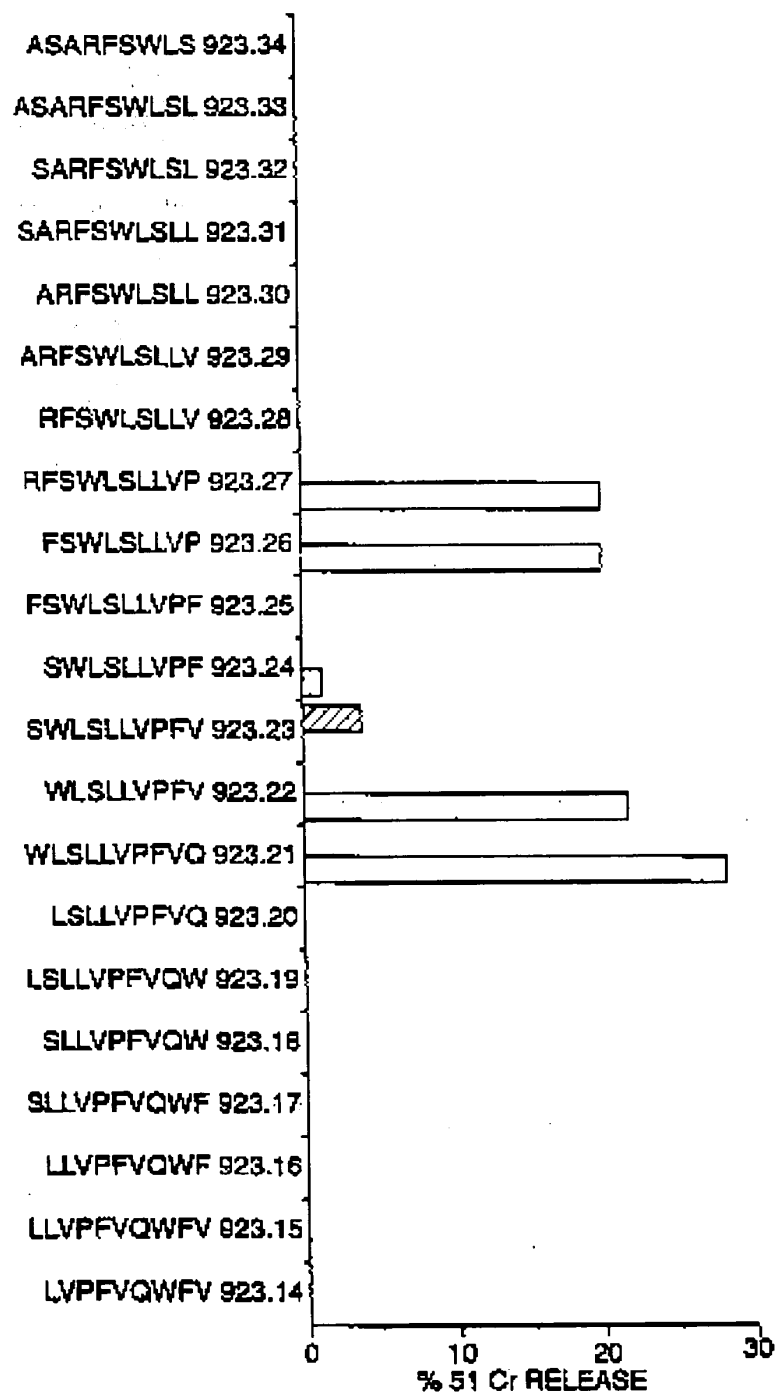


FIG. 13b